Male plumage variation and its role in reproductive isolation between house sparrows (*Passer domesticus*) and Italian sparrows (*P. italiae*)

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A new method for quantifying colours of *Passer* sparrows using digital imaging in the field

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Centre for Ecological and Evolutionary Synthesis

Department of Biosciences
Faculty of Mathematics and Natural Sciences

University of Oslo

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Part I

Male plumage variation and its role in reproductive isolation between house sparrows (*Passer domesticus*) and Italian sparrows (*P. italiae*)

Abstract

Reproductive barriers between diverging taxa are necessary for speciation to occur. Premating barriers are thought to be of especially great importance, such as plumage colour which is often used in species recognition and mate choice. In this study, I have investigated male plumage variation between a species of hybrid origin, the Italian sparrow (*Passer italiae*) and one of its parents, the house sparrow (*P. domesticus*) in their narrow hybrid zone in the Alps. The Italian sparrow is intermediate in plumage between the house sparrow and its other parent, the Spanish sparrow (*P. hispaniolensis*) but distinctively different from both, Hence plumage traits can potentially contribute to premating isolation between the hybrid and its parents. I use cline theory to infer selection on four sexually dimorphic plumage traits in males (crown, cheek, eyebrow and bib). Quantitative measurements of plumage traits obtained using digital photography were compared with a molecular hybrid index (species-informative SNPs) to compare changes in the plumage traits with genetic changes of a genome-wide average across the hybrid zone. I found crown colour to be under selection, supported by four indirect lines of evidence. First, crown colour was the most strongly species-diagnostic trait. Second, crown colour showed the narrowest cline. Third, if the crown had been neutral the cline width (of 33 km estimated here) would have required that the two species met only 43 generations ago, which is highly unlikely. Finally, estimates of strength of selection pointed out crown to be under strongest selection, with a likely 1-10% drop in fitness of intermediates. The other plumage traits did not seem to be under the same selection pressure, although a significant shift in cheek cline centre was detected, possibly indicating asymmetric female preference in favour of house sparrow cheek colour. Overall, this investigation reveals differential selection pressures among plumage traits across the Alps hybrid zone, where selection against individuals with intermediate crown colour most likely plays a role in premating reproductive isolation in this species complex, thereby contributing to homoploid hybrid speciation.

Introduction

The evolutionary processes responsible for the formation of species have interested biologists for decades (Darwin, 1859; Seehausen et al., 2014, in press). According to the biological species concept, speciation is completed when two divergent populations no longer interbreed and produce fertile offspring (Mayr, 1942). The most common form of speciation occurs when new species arise from the divergence of a single lineage. Another less recognized mechanism is hybrid speciation, where two species hybridize and give rise to a new taxon (Mallet, 2007). In either type of speciation, reproductive barriers must be present to prevent the homogenizing effects of gene flow and keep the divergent taxa distinct from each other. Thus, understanding the nature of reproductive isolation is essential in understanding the origin of new species. In principle, reproductive isolation between two taxa may result from pre- or postmating prezygotic barriers, or postzygotic barriers (e.g. sterility and inviability of hybrids) (Coyne & Orr, 2004). Premating reproductive barriers are thought to be the primary mechanisms of reproductive isolation between taxa (Seehausen et al., 2014, in press), and in this thesis I investigate premating isolation and its role in the process of speciation.

Premating barriers inhibit reproduction before mating takes place, and can further be divided into habitat, temporal and behavioural isolation. The first two barriers reduce the probability of encountering a mate of the other species by adaptations or preferences to different habitat types or breeding times, respectively (Coyne & Orr, 2004). Sexual isolation consists of traits that reduce interspecific sexual attraction between sexes, thus impeding courtship and mating between them. Structural or behavioural signals such as colour displays, song or pheromones are typically used by females to identify potential mates (Baker & Baker, 1990; Seehausen & van Alphen, 1998; Smadja & Ganem, 2002). In this process recognising conspecifics from heterospecifics is important for females as heterospecific matings often yield offspring of lower viability, thus wasting the investment of producing and rearing of young (Trivers, 1979; Andersson, 1994; Coyne & Orr, 2004). The male signals and female preferences for those signals together constitute the mate-recognition system, and they may diverge between populations as a result of genetic mechanisms such as genetic drift, sexual or natural selection, due to non-genetic

mechanisms such as learning (Servedio et al. 2007), or combinations of these factors (Andersson, 1994; Coyne & Orr, 2004; Servedio et al., 2007). For example, *Heliconius* butterflies exhibit a great variety of colour patterns within and among species that have evolved by natural selection to mimic each other's colours to avoid predation (Jiggins et al., 1996). In these species mate choice is also based on colour, and wing colour patterns are important mate recognition cues leading to assortative mating (Mallet et al., 1990; Jiggins et al., 2001; Jiggins et al., 2004). Sexual barriers are often inferred from controlled mate choice experiments including no-choice tests (e. g. one heterospecific female presented with one male) or with-choice tests (e. g. one female of each species presented simultaneously with one male of each species) to reveal non-random mating patterns (Rutstein et al., 2007). Such experiments could also include artificial changes of signalling traits to observe the effects on interspecific mating, such as changing the species-specific colour patterns (e. g. Wiernasz & Kingsolver, 1992), exposing females to song from different species using loudspeakers (e. g. de Kort & ten Cate, 2001; Irwin et al., 2001; Safi et al., 2006) or manipulating the light environment (Seehausen & van Alphen, 1998; Summers et al., 1999). However, controlled mate-choice experiments are not feasible for all taxa as they depend on a species' nature and behaviour. In such cases, other indirect methods for detecting premating barriers are necessary.

Hybrid zones, areas where genetically diverged populations meet, interbreed and produce hybrids, are valuable for studying speciation and reproductive isolation (Hewitt, 1988; Harrison, 1993). The semi-permeable nature of hybrid zones allows gene flow for neutral traits but restricts movement of genes under disruptive selection, thus allowing for identification of traits and underlying genes contributing to reproductive isolation. Hence, these zones provide natural laboratories for studies of organisms which are difficult to breed or manipulate in captivity. Much of the knowledge concerning hybrid zone patterns is retrieved from Barton and Hewitt's cline theory (Barton & Hewitt, 1985, 1989). They argue that most hybrid zones are "tension zones" in which the hybrid zone constitutes a cline maintained by the balance between dispersal of parental types into a hybrid zone and selection against hybrid types. The two forces create a "tension" keeping the zone in balance. Selection can be exogenous or endogenous, meaning that alleles are selected against in a new habitat or in the novel genetic background of the parental taxa (Hewitt,

1988). In geographical cline analysis, the clines may vary along a geographical transect in two parameters - width and position, which can be used to infer if there is selection against hybrids, and to compare the strength of selection between traits or loci (e.g. Gay, et al., 2008; Teeter et al., 2008). When all clines exhibit approximately the same widths (concordant clines) and clines centre positions (coincident clines) this indicates similar magnitudes of selection on all traits represented by those clines and provides evidence for strong genome-wide selection. In contrast, variation in cline widths and centres indicates that traits are reacting differently to selective pressures. The cline width is determined by the strength of selection against hybrids relative to average lifetime dispersal; weaker selection produces a wider cline and stronger selection produces a narrower cline, and non-coincident cline centres may indicate differential introgression into the other species' range. In this investigation I use geographical cline analysis as a tool for investigating if there is selection against intermediate male plumage traits in a hybrid zone between a hybrid species, the Italian sparrow (*Passer italiae*) and one of its parental species, the house sparrow (*P. domesticus*). Plumage differences might play a significant role in species recognition (Mayr, 1942; Sætre et al., 1997; Price, 2008), and thus constitute a potential premating barrier between the Italian and the house sparrow.

The Italian sparrow represents one of few documented cases of animal homoploid hybrid speciation (HHS) (Elgvin et al., 2011; Hermansen et al., 2011). This is the form of speciation in which a novel species emerges from a hybridization event without a change in chromosome number (Coyne & Orr, 2004; James Mallet, 2007). The Italian sparrow originated from interbreeding between house sparrows and Spanish sparrows (*P. hispaniolensis*) in the past (Elgvin et al., 2011; Hermansen et al., 2011; Trier et al., 2014). This study system is well suited for investigating the evolution of reproductive isolation because the hybrid species is still in direct contact with both parental species. Italian sparrows are present over the entire Italian Peninsula as well as some of the surrounding Mediterranean islands. They occur in sympatry with Spanish sparrows on the Gargano peninsula in the south of Italy, and meet house sparrows in the Alps in northern Italy in a narrow hybrid zone (Figure 1). Interestingly, there is limited gene flow between the hybrid species and its parents. There is no documented inbreeding between Italian and Spanish sparrows in Gargano, and apparently only restricted gene exchange with house sparrows in the Alps hybrid zone (Hermansen et

al., 2011; Trier et al., 2014). This suggests the presence of reproductive barriers impeding backcrossing with parental taxa. Male plumage variation in this sexually dimorphic species complex may represent premating barriers as the Italian plumage pattern is distinct from either of the parental species, albeit intermediate between the two (Meise, 1936; Summers-Smith, 1988) (Figure 1). One striking plumage difference is the colour of the crown, which is slate grey in house sparrows and chestnut brown in Spanish and Italian sparrows. Further, the Italian sparrow has white cheeks similar to the Spanish sparrow and a distinct white area above the eye (here further referred to as the "eyebrow"), which appears less conspicuous in house sparrows. The eyebrow is often depicted as larger in Spanish than in Italian sparrow in field guides, however quantitative measures are lacking. Italian sparrows are more similar to house sparrows in having rather small black bibs with grey flanking (although Italian males have larger bibs in southern Italy and some black flanking in Sicily) (Hermansen et al., 2011). The Spanish sparrow male exhibits a huge black bib with large black flanks along the belly. The colour of the back also differs between male Spanish sparrows (cream and black) on one side and Italian and house sparrows (brown and black) on the other. However, quantitative measures of plumage differences has been lacking and the description above is thus qualitative in nature, largely based on information from field guides and other books (e.g. Summers-Smith, 1988; Anderson, 2006).

The hybrid zone in northern Italy runs from the coastal border between France and Italy in the west, following the ridge of the Alps along the Italian border with Switzerland, Austria and Slovenia to the Adriatic coast, and is found at altitudes between 500-1500 metres (Summers-Smith, 1988). Sparrows of Italian phenotype (largely based on crown colour) are found to the south and house sparrow phenotypes to the north of the alpine ridge. Even though the high mountains reduce movement to some extent as they are covered with snow most of the year, there are intermediate hybrid phenotypes in the middle of the hybrid zone (Summers-Smith, 1988; Hermansen et al., 2011), which lies on the southern slopes of the Alpine ridge in this region. Not only is the hybrid zone narrow in extent (approximately 30-40 km wide), but is also reported to be stable over time (Summers-Smith, 1988). This indicates that there are some factors, environmental, behavioural or both, maintaining the separation of the two sparrows that prevent them from moving into each other's range. Premating barriers (linked to plumage differences), geography (elevation preferences) and ecology (e.

g. beak differences) are suggested as potential contributors to reduced gene flow (Summers-Smith, 1988). However, here I focus on the potential role of male plumage colour.

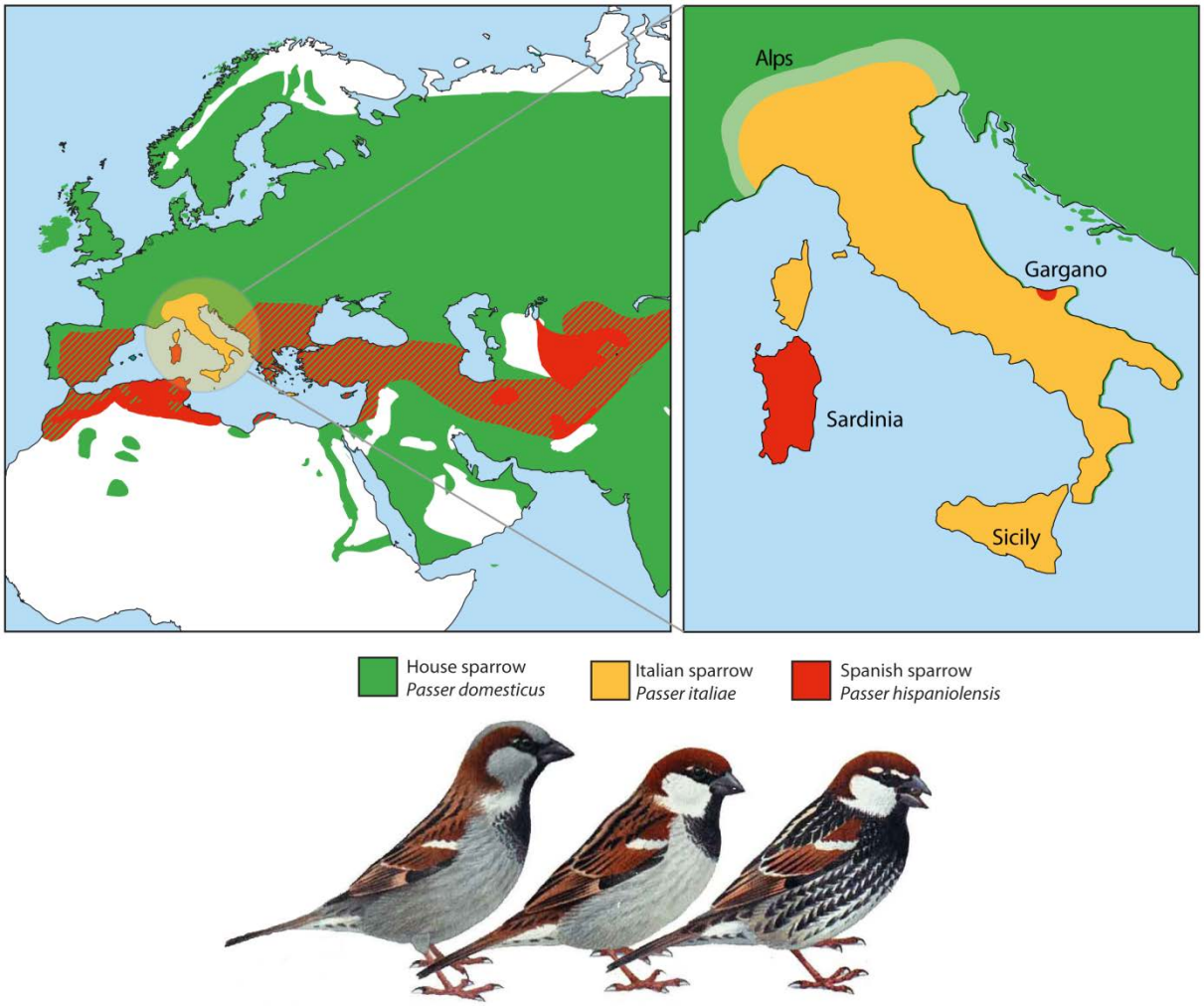


Figure 1 The *Passer* study system Above: Distribution map of Spanish sparrow (*P. hispaniolensis*), Italian sparrow (*P. italiae*) and house sparrow (*P. domesticus*). Below: Male plumage colouration differences between the two parental taxa and the hybrid species showing the hybrid Italian sparrow in the middle.

To date there has been little focus on premating barriers such as sexual signals and mate choice in research on HHS. In this form of speciation, reproductive isolation is thought often to arise through ecological transgression (Rieseberg et al., 1999; Mallet, 2007), in which novel adaptions in the hybrids allow them to exploit niches unavailable to the parental taxa, in which case sexual signals may not be important. However, the Italian sparrow appears to

be ecologically very similar to the house sparrow (Hermansen et al., 2011; Trier et al., 2014), thus warranting investigation of the potential role of sexual isolation.

Here, I investigate variation and differentiation in four male plumage traits: crown, cheek, eyebrow and bib, between the Italian sparrow and the house sparrow across the Alps hybrid zone. These are male-specific secondary sexual traits, which are likely to play a role in mating. Crown colour is known to be a species-diagnostic trait and thus likely to be involved in species recognition. My first aim is to investigate to what extent any of the other plumage traits (cheek, eyebrow and bib) are species-diagnostic for Italian and house sparrows, and are thus traits potentially involved in species-recognition.

My second objective is to investigate whether the pattern of variation in plumage traits is consistent with them being under selection, and if the more species-diagnostic traits show stronger signs of selection, as would be expected if species recognition contributes to isolation. The structure of the hybrid zone (narrow, stable and with intermediate phenotypes in the centre) suggests that the zone may be a tension zone maintained by selection against hybrids (Barton & Hewitt,1985; Barton & Gale, 1993; Baird & Macholan, 2012). When comparing sparrow plumage trait clines with a molecular hybrid index representing a genome wide average, I can look for deviations from cline concordance and coincidence to infer selection. A narrower cline for any of the plumage traits than the cline for hybrid index would indicate selection against phenotypic intermediates at those traits potentially due to female preference for conspecific phenotypes. Hybrid zone clines often exhibit a sigmoid shape (s-formed), which could arise as two species meet. However, over time without selection, neutral diffusion will flatten the cline as the genes of the two species move into each other's range (Hewitt, 1988). Thus, if there is no selection on male plumage traits, I will expect to find clines broader and flatter than the hybrid index.

Materials and methods

Study site

Sparrows were caught using mist nets at 35 locations in Italy and Switzerland during March, April and July 2012 (Figure 2 and Appendix 1). The approximate location of the hybrid zone between house and Italian sparrows was identified prior to sampling using crown colour, as previously done by others (Summers-Smith, 1988). Male birds with house sparrow phenotype were caught from north of the main Alpine ridge (n= 19), Italian sparrow phenotypes from the northern part of the Italian peninsula, close to Lake Como and Lake Maggiore (n= 56), and putative hybrids from the contact zone in the Alps (n=63). Three different groups of researchers did the sampling, led by Glenn-Peter Sætre (GlennSpring) and Richard Bailey (RichardSpring and RichardJuly) respectively. We measured the length and height of the "eyebrow" (see Figure 1) and took digital photographs for all other plumage measurements. We also took blood samples for genetic analysis. Permissions for sampling birds were obtained from the appropriate authorities in Italy and Switzerland.

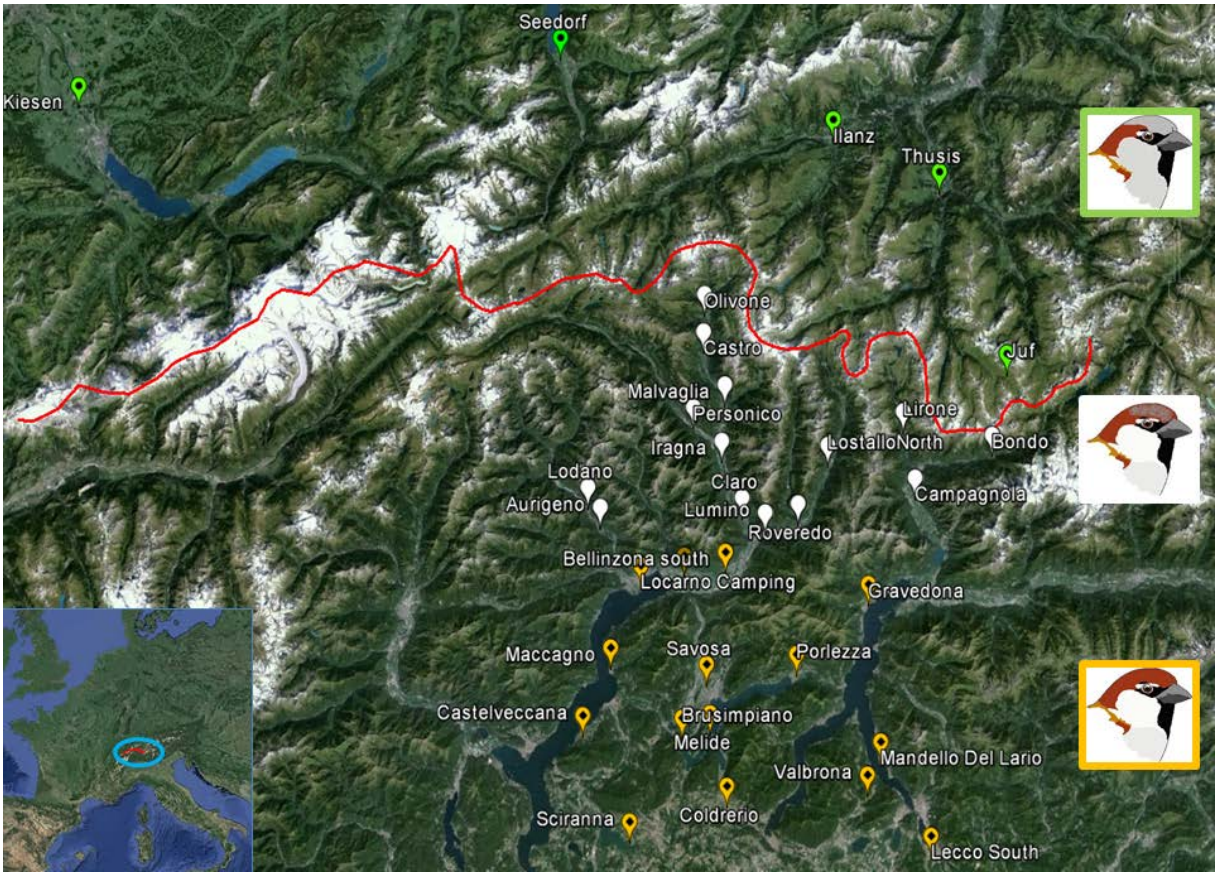


Figure 2 Sample locations of sparrows in the Alps hybrid zone (Google Earth, 2014). The droplets indicate

Figure 2 cont. locations where birds were sampled. Green, white and yellow droplets indicate house, hybrid and Italian sparrow phenotypes, respectively. The locations are plotted as longitude and latitude in signed decimal format. The red line indicates the location of the alpine ridge.

Quantification of plumage variation

I quantified variation in male plumage traits using size and/or colour measurements depending on each trait. Colour and size were combined in one case (eyebrow) and only size measurements were used in another (bib). The crown colour was quantified in the RGB model, to differentiate the species and to provide trait values for cline analysis. The RGB system is composed of the three primary colours, red, green and blue, which are grouped together in various ways producing the complete range of colours visible to the human eye. This system is widely used in quantifying animal colouration (e. g. Stevens et al., 2007; Bergman & Beehner, 2008; Runemark & Svensson, 2012). I also used luminosity, a measure of how dark or light the image is, for the other plumage colour measurements.

Equipment

Digital images were taken using a high resolution camera (Nikon D500, 16.2 megapixels) with a Sigma EM-140 DG Macro ring flash and a Sigma 50 mm 1:28 DG Macro lens, mounted in a standardized position (Figure 3d). The base was covered with millimetre paper background.

All captured birds were photographed from three different angles (dorsal, ventral and portrait; Figure 3a-c), and the images were saved in RAW format (NEF-files) as recommended by Stevens et al. (2007). Only males were used in this thesis as the sparrows investigated are sexually dimorphic with males being the sex showing plumage variation.

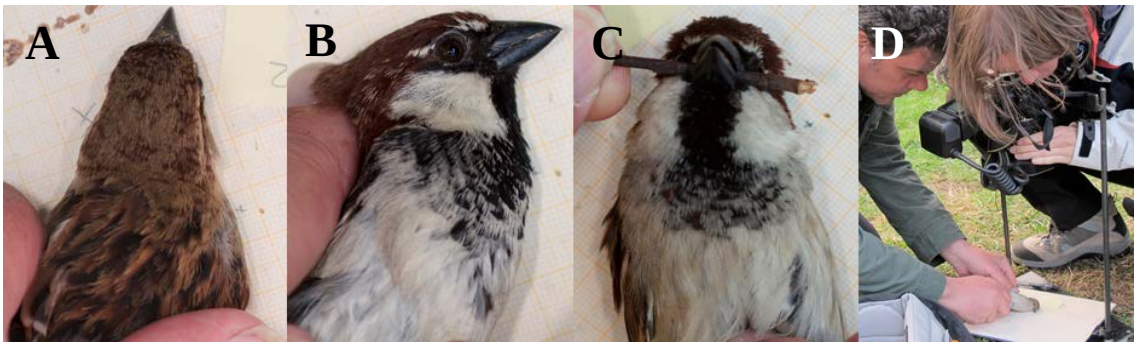


Figure 3 Example images of sparrows photographed at three different angles (a-c) and the photo set-up during field work (d).

Light standardization

Light variation among photos may affect the colour measurements and must therefore be standardised prior to measurement. After attempting numerous methods for standardizing the light across all photos, I found Adobe Photoshop® image-editing software, (Adobe Systems Inc., 2013) and a function called Match Colour to be the most effective. Match Colour works by matching all colours of a certain image (the target) to the colours of a standard reference photo (the source). Photo 674.NEF taken by the GlennSpring group was selected as the source image since the image contained the entire range of colours of interest – crown grey, yellow winter plumage, brown and white feathers (see below). All photos were manually matched after this reference photo to achieve a standardized set of photos. No other features in the images were altered.

Photos in RAW-format (NEF-files) require long loading times and storage space, thus increasing processing time and potentially causing a program-collapse when loading the resulting large-sized dataset. In this investigation, I measured large areas of the birds, obtaining mean and standard deviation of red, green and blue (RGB) and luminosity values across several hundreds of pixels. To overcome the technical difficulties with large image files, all NEF-files were converted to JPEG-files using the program ContentaConverter (ContentaConverter.com, 2013) and TIFF-files (larger files than JPEGs) directly from Photoshop. The conversion from raw-format to an image-format of fewer pixels (such as JPEG-format) should not affect the quality of the retrieved colour data (Bergman & Beehner, 2008).

After standardizing the light of all images and before colour extractions, a second quality check was performed by qualitatively assessing the photos based on exposure and focus. Only extremely dark or out of focus photos were removed at this stage to maximize sample size.

Crown

The objective was to estimate the proportion of house sparrow grey (HSG) versus Italian sparrow brown (IB) in the crown of all birds in the filtered data set.

Colour measurements for crown were conducted in three programs: Adobe Photoshop®, the commercial software package MATLAB (MathWorks Inc., 2000) and the statistical software R (R Core Team, 2013). Dorsal images were used (Figure 3a).

The birds were captured in early spring and during the summer. As many of the early caught birds retained variable amounts of winter plumage on the crown at capture I needed to make corrections. The slate grey crown of a house sparrow could easily be confused with the more yellow-grey winter plumage found in Italian sparrow crowns at the time of sampling. This was especially confusing in the hybrid zone where partly grey and brown crowns were observed. To ensure correct crown colour quantification, I used discriminant function analysis (DFA) to classify all pixels into three different crown colour categories – Italian brown (IB), house sparrow grey (HSG) and winter plumage (WP). All pixels categorized as WP were removed and the remaining pixels were used in the statistical analysis of crown colour variation.

RGB values of all pixels of a crown selection were needed to categorize each of them into one of the three colour classes, IB, HSG or WP. This was achieved by using a Matlab script written by Mikkel Brydegaard for this purpose (modification of script used in Runemark et al., 2010). The script enabled magnification of the crown area, and allowed a polygonal selection of the crown followed by an automatic download of the RGB values per pixel in the selection. Each individual's large matrix of RGB values could then be opened as a dataset in Matlab, copied into to a text program and saved as a *.txt –file, ready for classification analysis.

The DFA was performed in R using a linear discriminant analysis (LDA) function from the MASS package (Venables & Ripley, 2002). The training set, used as the reference for classifying all individual crown selections, was made in Adobe Photoshop using the colour corrected JPEG files. Twelve house sparrows and twelve Italian sparrows were selected from areas outside the hybrid zone to retrieve colour values for house sparrow grey (sample sites Andeer, Thusis, Kiesen, Ilanz, Juf and Seedorf) and for winter plumage and Italian brown (Lecco South, Sciranna, Porlezza, Valbrona, Castel Veccana, Chieve and Mandello). Italian sparrows do not have any grey in their crowns and are therefore ideal as the winter plumage reference. The reference selection included images from all three field groups and included images of different quality (e. g. focused and blurry photos), in order to incorporate likely variation in photo quality for birds to be classified. Two photos per individual were selected. In Photoshop, the *maximizer* tool was used to zoom down to pixel level to see a single feather barb and avoid shadows and black areas. The area of the colour of interest was selected using the "lasso"-tool allowing for free-hand selection, and the RGB levels were recorded using the histogram palette which shows the average RGB values over the selected area. This method is widely used in the colour quantification literature (Villafuerte & Negro, 1998; McGraw et al., 2002; Yam & Papadakis, 2004; Setchell et al., 2006; Bergman & Beehner, 2008). Each measurement was repeated three times per photo at randomly selected areas of the crown to account for variation. All selections were saved in the image-file, which again was stored as a Photoshop file (*.psd).

For the training set describe above, the leave-one-out cross validation approach of LDA classified the pixels to the three different colour categories (IB, HSG, WP) with 95% accuracy.

Having ensured a high degree of accuracy in colour classification of the training set, DFA was then used to classify all individual crown pixels in the JPEG images of all birds in the filtered data set (this included the birds in the training set plus the hybrid zone). Whereas only small areas of the crown were selected and analysed for the training set analysis, to ensure pure colours only, I selected the whole crown for pixel classification of the total data set. I took care to avoid including different-coloured areas around the edge of the crown. Each pixel is assigned a posterior probability of belonging to each of the three colours. To ensure a classification of good quality, only pixels with high posterior probabilities (pp) >0.7 were retained from the classification analysis, indicating that they were assigned to a specific

colour with high confidence. Pixels of lower quality (pp<0.7 for all categories) were removed from the analysis. The number of pixels classified in each category per individual was used to calculate percentage of grey per crown, HSG / (IB+HSG), leaving out all WP classified pixels. After good quality pixels were classified and proportion of grey calculated, all the images remaining were visually compared with their respective calculations of grey, and graded qualitatively according to whether the classification appeared to have worked successfully. A few photos (N=9) that visually appeared very different from the proportion of grey calculations were removed. I removed one additional individual because its eyebrow height value was missing. After the 3-step quality check, 139 individuals remained, and these same individuals were used for all other traits and analyses.

Cheek and eyebrow

The length and height of the eyebrow was measured in the field for each male bird using a dial caliper to the nearest 0.1 mm. These measures were combined with colour measures in the analysis.

Colour measurements for cheek and eyebrow were conducted in Adobe Photoshop and the statistical software R, and portrait images (Figure 3b) as match-coloured TIFF files were used.

Sparrow cheeks and eyebrows consist of neutral colours (black, grey and white)(Figure 1) and separate measurements of the three colours RGB would not have added much information in this case, as the three colours change proportionally when colour changes are neutral**.** The mean and standard deviation in luminosity per trait of interest was instead used as a measurement of colour variation for cheek and eyebrow. Measurements were therefore highly sensitive to variation in ambient light. Despite using Match Colours to standardize both colour and light variation among photos, some unwanted residual variation may have remained caused by light variation in the original photos. To remove any influence of such variation, the white millimetre paper was used as a standard reference to measure photo luminosity, and this was corrected for in the cheek and eyebrow measurements. Photo luminosity measurements were made using both the original uncorrected raw files and the match-coloured JPEG files, in order to test which was most strongly correlated with variation in cheek and eyebrow luminosity. Image luminosity was obtained by measuring the

luminosity of as large as possible areas of white background paper. In Photoshop, the cheek and eyebrow areas were selected using the "lasso"-tool and the histogram palette as described above, and RGB and luminosity were recorded. The linear regression analyses for plumage trait luminosity against photo luminosity were performed in R and the residuals for each trait were stored as separate datasets. Plumage showed a stronger relationship with luminosity of the original raw photos (see Results section), indicating that this explained more of the error in plumage luminosity measures. Hence these new, light-independent measurements of cheek and eyebrow were used in the statistical analysis.

Bib

The sparrow bib is uniformly black, and hence I examined variation in bib size rather than colour.

The bib measurements were done by visually investigating the portrait and ventral images (Figure 3b and c) of each individual. The bib size was ranked in two ways: one value was given for bib depth ↕ and another for bib width ↔. We use an image chart for scoring sparrow bibs in the field with values from 1-10, where 1 is very small (like a tree sparrow) and 10 is extremely large with large flanking (like an impressive Spanish sparrow) (Figure 1). Since this thesis only treats house sparrows and Italian sparrows in northern Italy, where very large bibs and black flanking are not expected to occur, I modified this chart and made one specifically for these two species (Figure 4(i)). Depth was ranked from 1 to 5, where 1 is the shallowest and 5 the deepest bibs. As there was less variation in bib width, I ranked them from 1 to 3, where 1 were narrow and 3 broad, reaching from shoulder to shoulder (Figure 4(i)).

The bib rankings were done two times to account for measurement variation. However, as the view of the bibs varied in different images, it was somewhat difficult to rank them. Even though all rankings were performed by the same person, the repeatability was lower than desired (correlation coefficient < 0.9) thus introducing a potential error. To still capture the variation in bib size and reduce error, the bib scorings were grouped into three classes for depth and three classes for width (small, medium and large) (Figure 4(ii)). For example, if a bib's depth was first ranked as 1 and in the next session ranked as 2 it was placed in the

group called 1-2. On the other hand, if a bib's depth was ranked as 1 in all sessions it was placed in the group called 1. Both these examples were finally grouped as a small (S) bib depth. For simplicity, the final small, medium and large sizes of bib depth and width were annotated the numbers 1, 2 and 3, respectively, and the bib was represented by two independent variables in the later analysis.

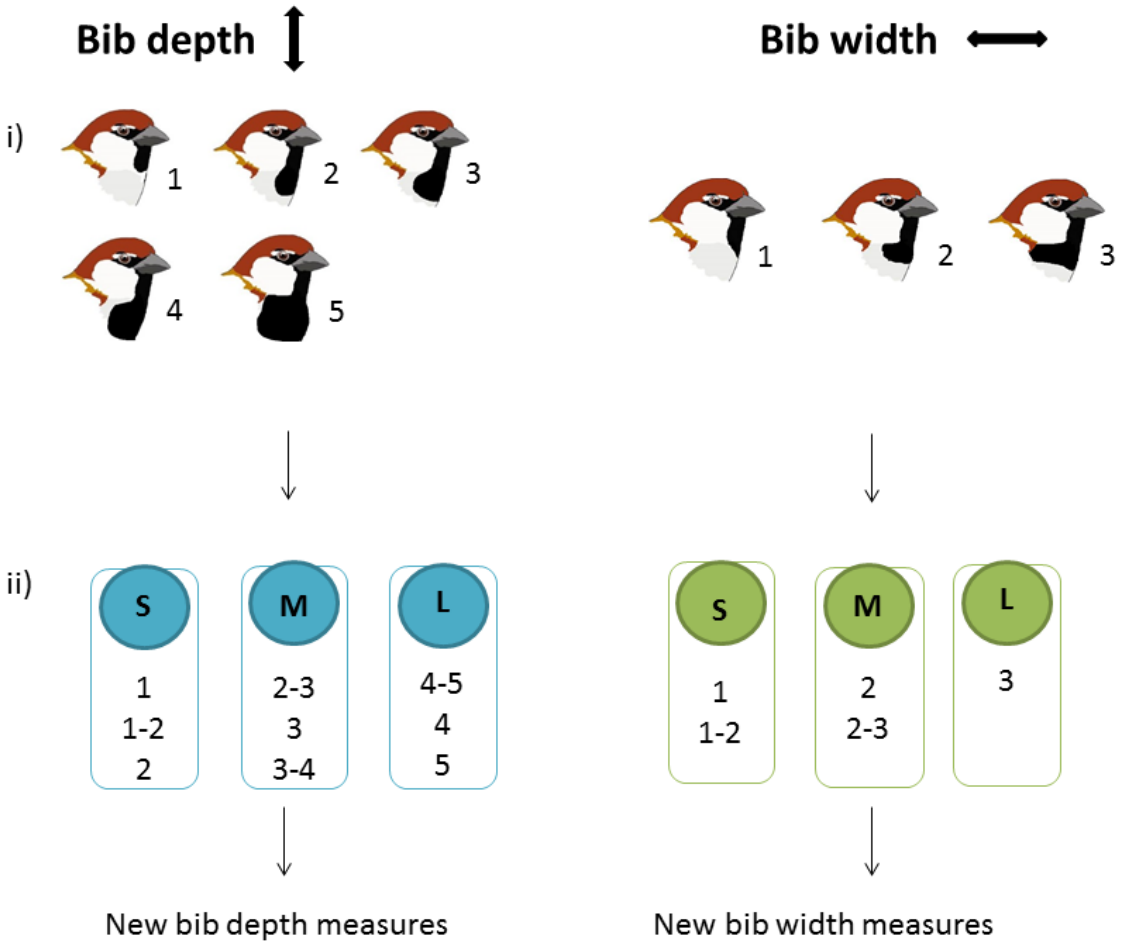


Figure 4 Two-stage bib measurement set-up used to rank bib sizes of sparrows. i) The bib scoring charts used in ranking bib depth and bib width per individual. ii) The bib rankings were further classified into three groups to account for measurement variation. If a bib was assigned the same score in different repeatability ranking sessions (e. g. 1), it was grouped as a 1. If a bib was assigned two different bib scores in different repeatability sessions (e. g. 1 and then 2), it was grouped as a 1-2 bib. Both these two examples belong to the final group small (S).

Hybrid index

A molecular hybrid index was analysed alongside plumage traits, to compare changes in the plumage traits across the hybrid zone with genetic changes representing a genome-wide average. The hybrid index was produced using a set of species-informative SNPs from coding genes (see Trier et al., 2014). Being species-informative and from coding genes, this set of SNPs is likely to show stronger clinal patterns than the genome as a whole, and indeed a number of the SNPs have been shown to be under selection in Italian sparrows, including in earlier collections in other parts of the Alps hybrid zone (Trier et al., 2014). This therefore represents a conservative standard genome-wide reference against which to compare plumage traits.

The hybrid index was constructed as in Trier et al. (2014). Three birds of each sex from each parental taxon were sent for transcriptome sequencing, and the isolated RNA was used to construct a cDNA library. Sequence alignment and mapping with the zebra finch genome gave a list of single nucleotide polymorphisms (SNPs) which was genotyped with the Sequenom MassArray system at CIGENE, Norwegian University of Life Sciences, Ås, Norway. After removal of SNPs with genotyping success < 97%, the final set of SNPs used was 81 (Appendix 2). The molecular hybrid index was calculated based on genotyping of the 139 birds used in colour analysis using these 81 SNPs. The index can be understood as the proportion of Spanish sparrow (relative to house sparrow) alleles ranging from 0 to 1. Hybrid index was calculated using the Introgress 1.2.3 package in R (Gompert & Buerkle, 2012).

Species diagnostic traits

Crown colour is the primary species diagnostic male plumage trait. Cheek, eyebrow and bib were investigated to infer if they too were species diagnostic for house sparrow and Italian sparrows. Canonical variate analysis (CVA) was performed to combine the multiple variables describing each plumage trait and to find the combination in which the difference between the two species was maximized. The CVA was done in R using the Morpho package (Schlager, 2013).

The choice of individuals for the parental reference set was based on the geographical distances from the main Alpine ridge for each location, measured in Google Earth (Google Inc., 2013) (Figure 2). All 35 locations were plotted into the online map program using their respective latitude and longitude coordinates. The alpine ridge was marked using the "*Add a path*" function which enables the creation of a continuous line connected by a number of dots of my choice. With the elevation scores visible in the Google Earth window, I created a line over the alpine ridge which separates the sampling locations. The actual distance between the alpine ridge and each location was measured using the "*Ruler*" function which provides a measurement of distance between two points in the Google Earth map. I measured the distance in a straight, vertical line (north-south). All measurements were done in kilometres and recorded from "*Map Length*" with "*Heading*" as close to 180° (north of ridge) or 0° (south of ridge) as possible. Distances south of the Alpine ridge were made negative to indicate direction. All distances are listed in Appendix 1.

All Italian sparrow individuals from the southernmost populations relative to the alpine ridge (Coldrerio, Sciranna, Melide, Brusimpiano, and Castel Veccana) were selected as Italian parental references. All individuals from the northernmost populations (Ilanz, Seedorf, Thusis, and Kiesen) were selected to serve as the parental reference for house sparrows. House sparrows from Juf were not included as they were too close to the ridge (4.5 km) on the northern side. These selections resulted in a parental reference set consisting of 17 Italian and 14 house sparrows. CVA was used for analysing cheek, eyebrow and bib since these plumage traits each had several variables describing them. Mahalanobis distances and p-values were calculated to determine the differences in traits between the two taxa. For crown and hybrid index, a t-test for unequal sample sizes was performed to test for significance in group mean difference between Italian and house sparrow parentals as a visual comparison with the other traits.

To retrieve a complete new dataset with the combined variables for cheek, eyebrow and bib for all individuals, LDA was performed using the MASS package in R based on the same parental reference set. The LDA resulted in LD1 scores for all individuals including hybrids, hence producing a single variable for each plumage trait, and the possibility to calculate parental means for each trait. LD1 scores are directly equivalent to CV1 scores. The scores and the parental means of cheek, eyebrow and bib were further used in cline analysis.

Cline analysis

I used cline analysis to infer possible selection against hybrid phenotypes for each trait, and to compare the plumage traits with each other and with the molecular hybrid index. Although we sampled in a two-dimensional manner, the samples sites of analysis were merged into a one-dimensional transect, ordered according to distance from the alpine ridge.

The cline analysis was performed in R and Excel (Microsoft Corporation, 2010) and was based on cline theory. A geographical cline model was used to describe the variation in plumage traits (crown, cheek, eyebrow and bib) and the hybrid index across the Alps hybrid zone. The pattern of trait variation was described by using a maximum likelihood method as in Teeter et al. (2008). The method uses a hyperbolic tangential (tanh) equation (1) to fit clines to the data and uses a search algorithm to find the maximum likelihood estimates of the cline parameters of interest.

(1) $$p = \frac{1}{2}[1 + \tanh\left(\frac{2[x-c]}{w}\right)]$$

In equation (1) *p*= trait value, *x*= geographic distance from alpine ridge, *c*= cline centre, defined as the point of the steepest slope or in other words, the point along the gradient where the plumage values change most rapidly, and *w*= width of cline, an estimate of the geographical distance where the change in trait values occur. The width is defined as the inverse of the maximum cline gradient, since this definition allows calculation of other quantities, such as strength of selection against hybrids (Barton & Gale, 1993).

In this investigation, a program similar to the cline fitting programs CLINEFIT (Porter et al., 1997), ANALYSE (Barton & Baird, 1995) and CFIT (Gay, et al., 2008) was developed by Dr. Richard Ian Bailey in the statistical platform R (see Bailey et al., 2012 for a previous version in another statistical software). Using this program, three cline parameters were estimated for all traits: centre, width and population standard deviation (i.e. the dispersion parameter). The parental means calculated earlier where used as p_{max} and p_{min} for each trait in the cline analysis. Transformation of the data was done prior to analysis to ensure a sufficiently high variance (>1, achieved by multiplying all values by 5) and to force the house sparrow parental mean to be larger than that of the Italian sparrow (achieved by inverting all values

to be negative followed by adding 10 to make the values above positive again, but now with the house sparrow mean being the largest). These neutral transformations were necessary for the cline program to run correctly. The confidence intervals for the parameter estimates were calculated in the cline fitting program using a Hessian matrix (matrix of second partial derivatives of the likelihood function, also known as Fisher's information matrix). This provides a measure of how steeply the likelihood surface drops off around the maximum likelihood estimate, and hence how informative the best estimate is. The square roots of the diagonal elements of an inverted negative Hessian matrix represent the standard errors of the estimated parameters. The 95% confidence intervals are then 1.96 x standard error. I used overlap of confidence intervals as a test of statistical significance among cline parameters of different traits. The centre and width maximum likelihood estimates given by the cline analysis were each plotted together for all traits.

Clines may also be the result of neutral introgression after secondary contact. Such clines are predicted to become wider at a specific rate over time (Hewitt, 1988). Given an estimate of dispersal and a probable time since contact, the actual cline width can be compared with an expected cline width to determine the probability that the actual clines are results of neutral diffusion. To further strengthen the analysis therefore, I compared the cline width estimates with artificial widths estimated from neutral diffusion as derived by Barton & Gale (1993) (2).

(2) $$w = 2.51\sigma\sqrt{T}$$

In this equation T = number of generations since secondary contact, and σ = average lifetime dispersal. The *Solver* function in Excel was used to estimate the number of generations (T) of neutral introgression since secondary contact required to produce the maximum likelihood cline width estimate for my plumage trait measurements. It is likely that the Italian and house sparrow came into contact some thousands of generations ago (Hermansen et al., 2011). Hence, if the neutral model provides much shorter time estimates selection can be inferred.

Estimating strength of selection

The strength of selection against hybrids can be calculated from the cline width estimate and an estimate of average lifetime dispersal. The width is always proportional to $l=\sigma/\sqrt{s}$, where l is the "characteristic scale of selection" (the minimum distance over which selection can change allele frequencies) and s is the selection coefficient. However the estimate varies somewhat depending on the mode of selection (Barton & Gale 1993). Given an assumption of disruptive selection against intermediate plumage, caused by assortative female choice, I used a rearrangement (4) of equation (3) from Barton & Gale (1993). After looking through the literature to find the most suitable equation, equation (3) was chosen as it can be used when selection acts on heterozygotes (Bazykin, 1969). Overall, the different equations available for the diverse types of selection are very similar in magnitude and would most likely give similar results or at least reveal large differences in selection strength between traits (Barton & Gale, 1993).

(3) $$w = \frac{2\sigma}{\sqrt{s}}$$

(4) $$s = \left(\frac{2\sigma}{w}\right)^2$$

The natal dispersal distance, the movement of an individual from birthplace to breeding place, for house sparrows was used as the variable σ in this analysis. The average dispersal distance was set to 2 km (based on a review in Anderson, 2006, and references therein). As this estimate does not include long-range dispersal and thus may be an underestimate, I also estimated selection and neutral introgression using lifetime dispersal estimates of 5 and 10 km.

Results

Results of regression analysis for cheek and eyebrow

A linear regression analysis on photo luminosity versus cheek and eyebrow luminosity, including both RAW and corrected comparisons, showed that RAW regression residuals were the best choice for light-independent measurements of cheek and eyebrow as the F- values were typically higher and the p-values were lower for RAW compared to match-coloured JPEG image files, and thus explained more of the variation (Table 1).

Table 1 Linear regression (*F*- and *p*-values) of photo luminosity (white paper background) and *Passer* sparrow cheek and eyebrow luminosity using RAW-file images and luminosity corrected JPEG-files. The eyebrow was often broken in two plumage parts, therefore the two measurements (front and rear) were tested separately.

		RAW-files		***Corrected JPEG-files***	
Trait	Mean of RGB	*p-value*	*F-value*	*p-value*	*F-value*
Cheek	Red	0.0036	9.374	0.0084	7.55
	Green	0.0057	8.376	0.0021	10.52
	Blue	0.0061	8.238	0.0020	10.72
Eyebrow front	Red	9.89e-06	24.52	0.0800	3.203
	Green	1.37e-05	23.57	0.0064	8.145
	Blue	9.89e-06	24.52	0.0058	8.341
Eyebrow rear	Red	9.522e-08	39.66	0.0044	8.957
	Green	4.352e-07	34.36	0.0003	15.37
	Blue	5.027e-06	26.54	0.0002	16.83

Species-specific plumage traits

The CVA and t-test showed that cheek, eyebrow and bib are species-informative plumage traits in the house sparrow and the Italian sparrow. The mahalanobis p-values retrieved from the CVA showed significant differences between the parentals in all plumage traits tested (Table 2). However, these plumage traits are not as species diagnostic as the crown, which always exhibits grey and brown colour respectively in house and Italian sparrows. T-tests were performed to compare group means between the two bird taxa. As expected, crown colour and hybrid index (HI) were highly significantly different (t= 29.87, df=20.4, p<2.2e-16 for crown and t=-8.14, df=28.1, p<5.7e-09 for HI). Figure 5 (a,b) shows the highly distinct crown colours and the slightly less distinct hybrid index when the respective trait values are plotted against frequency. The histograms retrieved from the CVA (Figure 5c-e) showed less distinct grouping of the other plumage characters with greater overlap of values between the two groups compared to crown colour and hybrid index. All plumage traits were significantly different between the house and the Italian sparrows suggesting that they could potentially be involved in species recognition, but the more diagnostic nature of the crown colour suggests it may be a more important character.

Table 2 Mahalanobis distances and p-values derived from canonical variate analysis testing for differences in traits between the house sparrow and the Italian sparrow near their hybrid zone in the Alps.

Trait	Mahalanobis	
	Distance	Probability
Cheek	1.526	0.001
Eyebrow	1.876	0.001
Bib	1.423	0.001

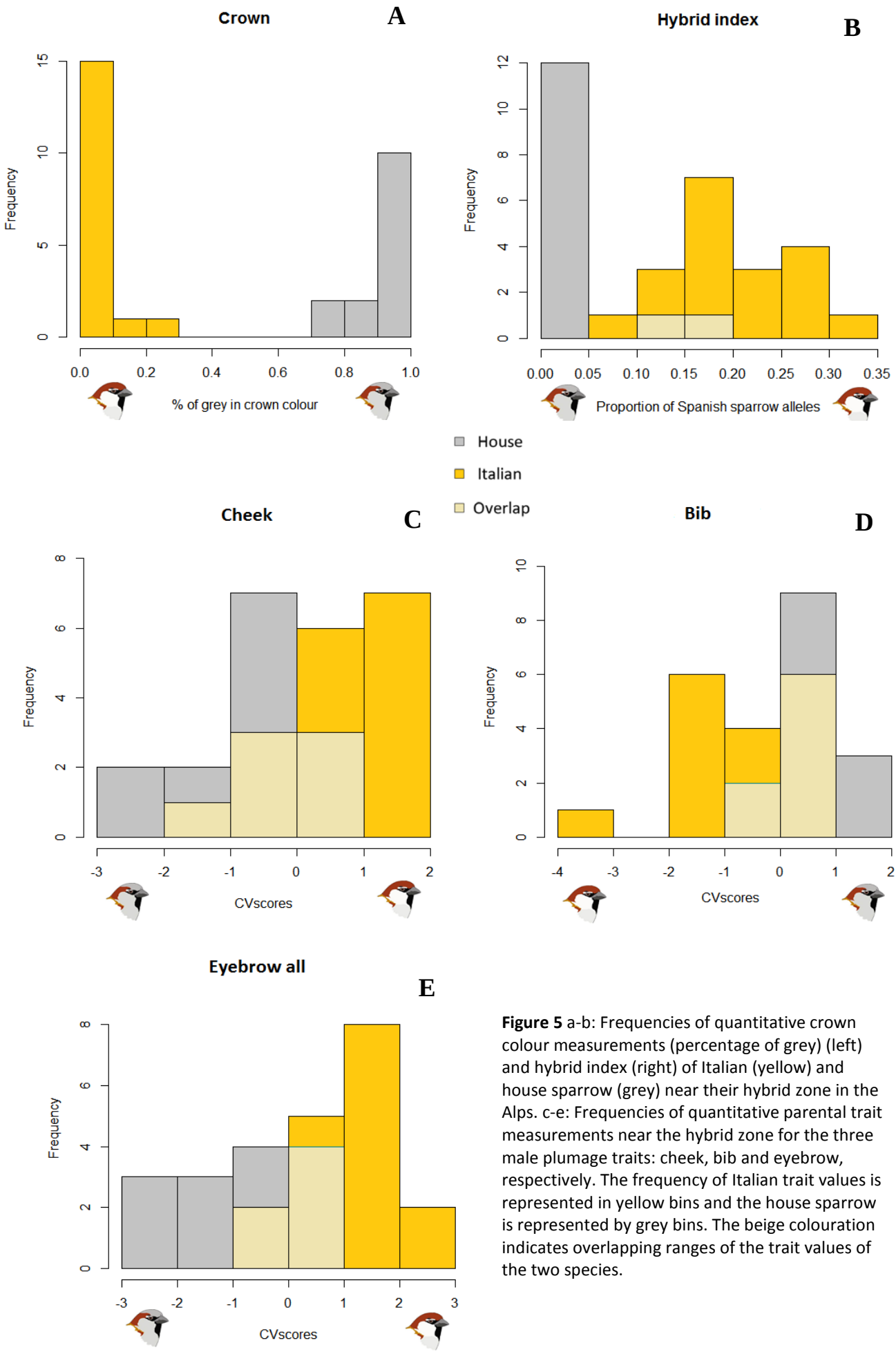


Figure 5 a-b: Frequencies of quantitative crown colour measurements (percentage of grey) (left) and hybrid index (right) of Italian (yellow) and house sparrow (grey) near their hybrid zone in the Alps. c-e: Frequencies of quantitative parental trait measurements near the hybrid zone for the three male plumage traits: cheek, bib and eyebrow, respectively. The frequency of Italian trait values is represented in yellow bins and the house sparrow is represented by grey bins. The beige colouration indicates overlapping ranges of the trait values of the two species.

Results of cline analysis and estimates of selection on plumage traits

The geographical cline analysis revealed crown colour as possessing the steepest cline with a lot of phenotypic variation in populations near the cline centre (Figure 6). Furthermore, the crown colour cline exhibited a narrow confidence interval (Table 3 and Figure 7a-b) showing a good fit of the data to the cline model used. The other male plumage colouration traits analysed (cheek, eyebrow and bib) did not show the same narrow clinal pattern and had larger confidence intervals (Figure 6b-e and Figure 7) indicating a poorer fit of the data to the model compared to crown colour. The cline for hybrid index was narrower than the cline for cheek, eyebrow and bib, but still not as narrow as the crown colour cline. The overlap of the confidence intervals (CI) was used to infer if crown colour was significantly different from hybrid index. The CI of crown colour does overlap with the CI of hybrid index implying that the difference is not statistically significant according to this criterion. However, using a less conservative criterion, crown CI does not overlap with the best estimate (the maximum likelihood estimate of width) of the hybrid index (Table 3 and Figure 7a-b).

I found evidence for a shift in cheek cline centre compared to the rest of the cline centres, the others being concordant (Figure 6f). The cheek centre was significantly shifted to the south as its confidence interval did not overlap with that of crown or HI, and only marginally with the eyebrow (Figure 7c-d, Table 3).

An estimate of the number of generations needed to reach the same cline width as retrieved from the neutral introgression analysis implied very short time since contact between the Italian and the house sparrow (Table 3). As this short time since contact seems very unlikely, the cline width of crown colour in particular appears to be narrower than can be explained by neutral processes alone, indicating that hybrid crown colours are likely to be selected against.

Selection coefficient estimates also suggest that crown colour is under stronger selection compared to the other male plumage traits (a minimum of s=0.014) (Table 3). Furthermore, the estimated strength of selection was an order of magnitude lower for the other plumage traits.

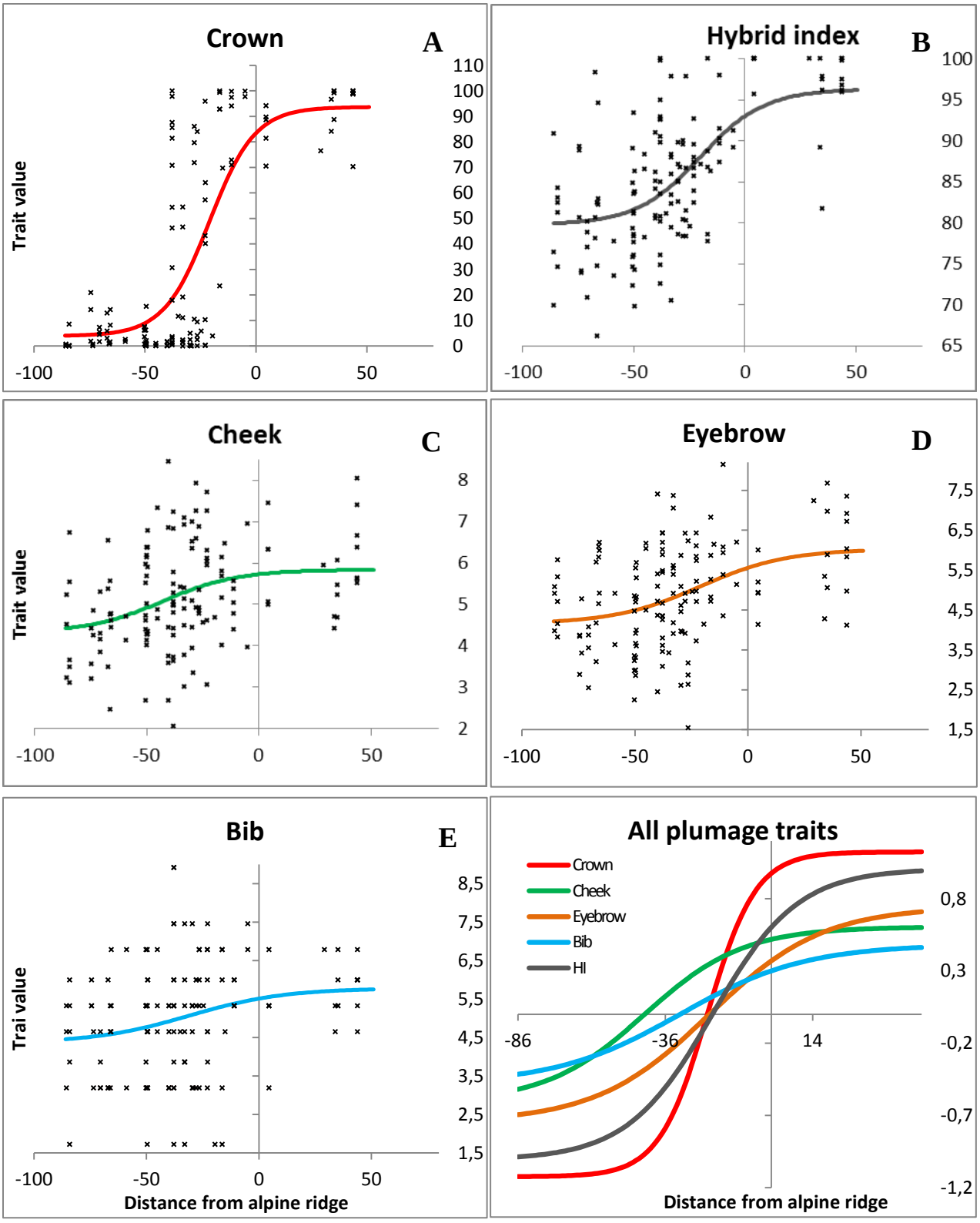


Figure 6 Geographical clines showing the change in male plumage traits and hybrid index across the hybrid zone between house and Italian sparrows in the Alps. Each cline is a maximum likelihood fitted curve, and the line represents the predicted mean based on the actual data points which are shown as small black crosses. The x-axis shows the geographical distance (km) from the alpine ridge separating the two taxa, and the y-axis shows the plumage trait value. The y-axis for each individual trait was scaled after the minimum and maximum trait values of the respective trait. A steep curve indicates selection against intermediate phenotypes. The bottom left figure shows all plumage traits and hybrid index clines together. The clines were scaled to fit the same axis by substracting the trait sample mean from the variables in the trait dataset (centring) followed by dividing the variables on the sample standard deviation (scaling) as in Schielzeth (2010).

Table 3 Overview of cline parameters with 95 % confidence intervals, selection coefficients for the plumage traits, and estimates of the generation time since contact necessary to reach the estimated cline widths if traits introgressed neutrally. The cline parameters (in kilometres) are the maximum likelihood (ML) estimates derived from cline analysis, and cline centres are shown in kilometres from the alpine ridge in which a negative value indicates distances south of the ridge and a positive value indicates distances north of the ridge. The ML value provides a measure of how well the cline equations fit the data. However the value is dependent on each dataset and is only included as a part of the statistics and not for comparisons among traits. Selection coefficients indicate strength of selection on each trait and are calculated based on three different estimations of dispersal distance of the house sparrow. The generation time estimates for reaching the same width derived from the cline analysis with neutral introgression were calculated using an equation using the same dispersal distances as with estimating strength of selection.

Trait	Width, ML (±CI)	Centre, ML (±CI)	ML value	Dispersal distance, σ (km/gen)	Selection coefficient, s	Gen. time estimate
Crown	32.78	-21.74	636.88	2	0.014	42.64
	(20.26, 45.30)	(-24.68, -18.79)		5	0.093	6.82
				10	0.372	1.71
Cheek	66.90	-42.71	218.44	2	0.004	177.6
	(12.94, 120.87)	(-54.44,-30.98)		5	0.022	28.42
				10	0.089	7.1
Eyebrow	76.51	-20.91	215.87	2	0.003	232.29
	(14.38, 138.65)	(-33.70,-8.12)		5	0.017	37.17
				10	0.068	9.29
Bib	84.86	-31.00	244.12	2	0.002	285.76
	(-6.79, 176.52)	(-49.46,-12.54)		5	0.014	45.72
				10	0.056	11.43
HI	57.91	-20.42	450.86			
	(33.70, 82.13)	(-26.70,-14.14)				

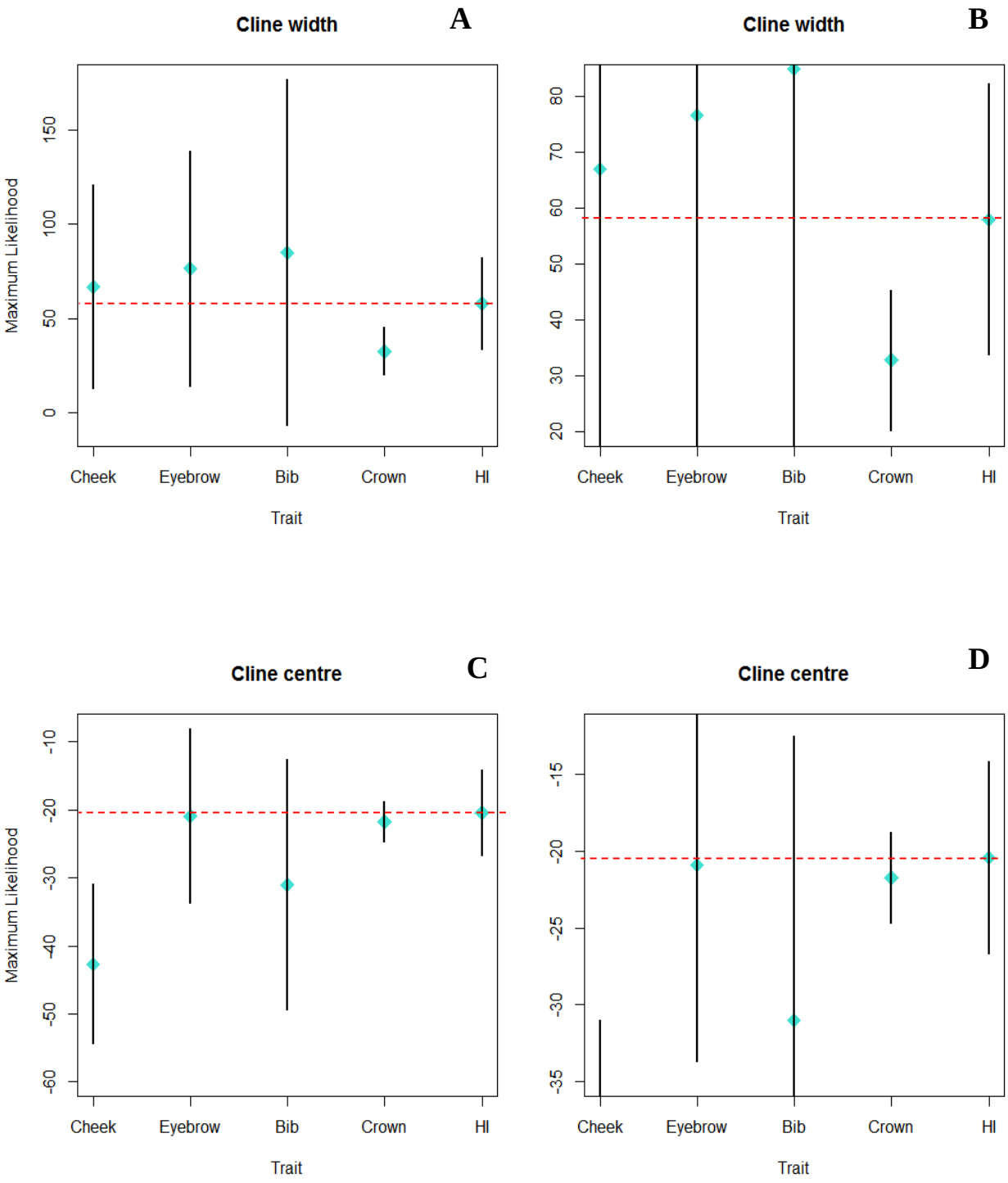


Figure 7 Estimates of cline width and centre from cline analysis of house and Italian sparrows and their hybrids in the Alps hybrid zone. The x-axis shows the different male plumage traits and hybrid index (HI). The y-axis shows the maximum likelihood estimates of either widths (a, b) or centres (c, d) retrieved from the cline analysis. Blue dots indicate the width or centre estimate with 95% confidence intervals. The red, broken line shows the best estimate (the maximum likelihood estimate of width or centre) of the hybrid index. Non-overlapping confidence intervals of traits indicate significant differences in cline estimates. The left graphs of the cline estimates are large scale plots showing the full range of cline widths and centres and the entire range of confidence intervals. The right figures are zoomed in to facilitate inspection of confidence interval overlaps.

Diagnostic properties of a trait and cline width

A species-diagnostic plumage trait is more likely to be involved in species recognition by female choice. I would therefore predict narrower cline widths of such traits compared to less diagnostic traits. I performed additional t-tests on the LD1 scores retrieved from the Linear Discriminant Analysis on the multivariate plumage traits cheek, eyebrow and bib to enable a comparison of cline widths and the measure of how diagnostic the traits are (the p-values) with crown colour and HI. The cline widths of the plumage traits showed a positive relationship with the p-values from the t-tests (Figure 8 and 9). Crown colour exhibited the narrowest cline, and even though the cheek cline width is deviating somewhat the overall pattern is one of increasing cline width with decreasing level of diagnostic properties.

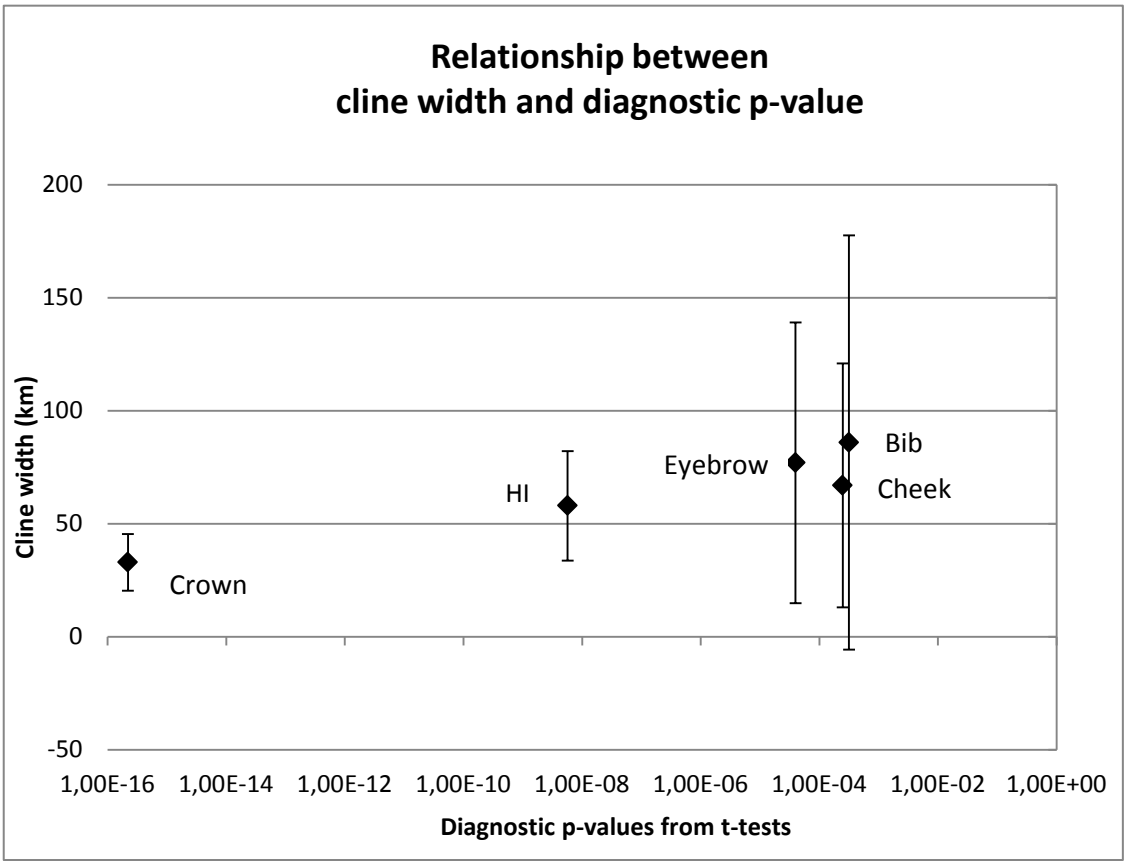


Figure 8 Relationship between cline width and species diagnostic property (p-values of population mean comparisons of house and Italian sparrows). The x-axis displays p-values from t-tests performed to establish a measure of how species diagnostic each trait was, plotted on a logarithmic scale, and the y-axis shows the maximum likelihood estimate of cline width retrieved from cline analysis of each plumage trait and hybrid index.

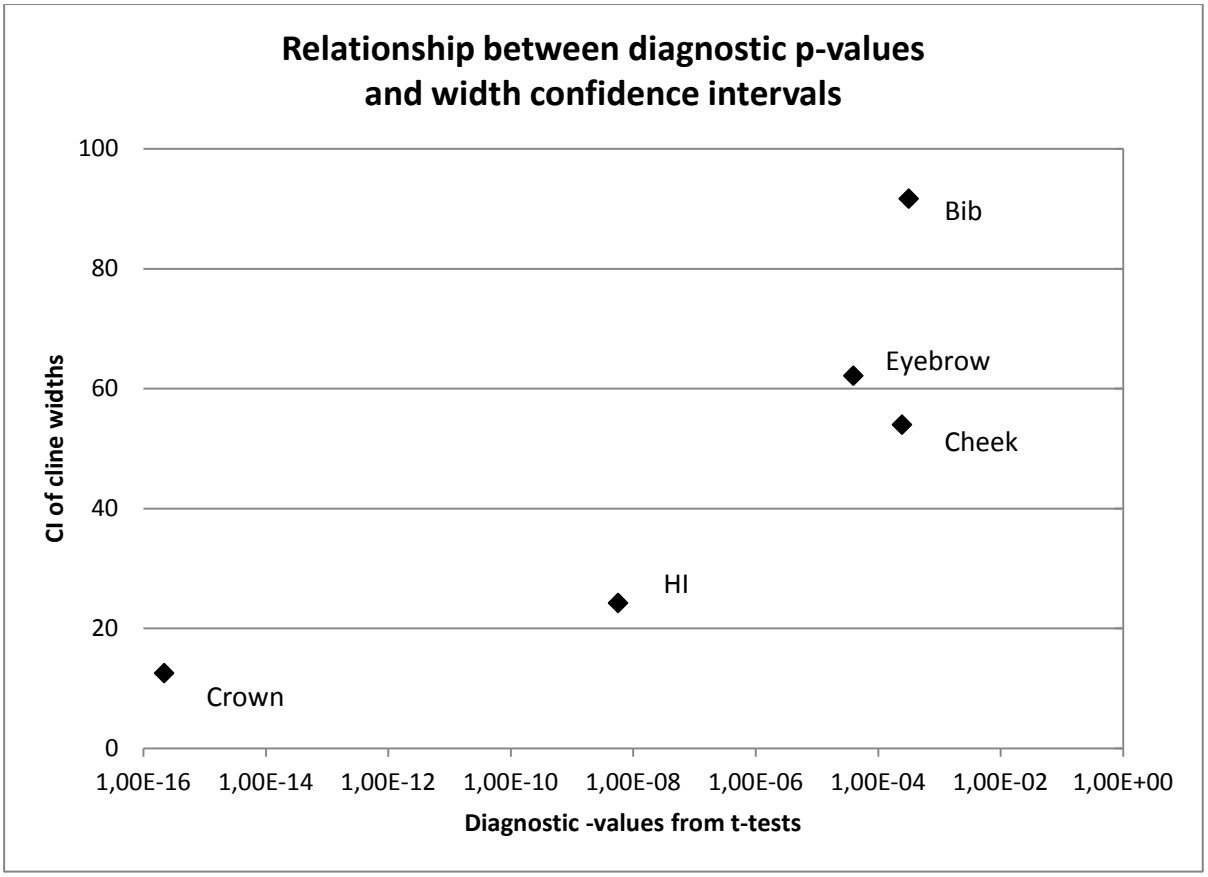


Figure 9 Relationship between 95% confidence intervals of cline width a nd species diagnostic property (p-value of population mean comparisons of house and Italian sparrows). The x-axis displays p-values from t-tests performed to establish how species diagnostic each trait was, plotted on a logarithmic scale, and the y-axis shows the 95% confidence interval retrieved from cline analysis for each plumage trait and hybrid index. The confidence interval is used to display how "clinal" the trait is as it is a measure of how well the data fit the cline model.

Discussion

In this thesis, I have investigated plumage traits as potential premating barriers between the hybrid Italian sparrow and one of its parents, the house sparrow, in the Alps hybrid zone by analysing the geographic pattern of variation in four male plumage colouration traits (crown, cheek, eyebrow and bib). I found cheek, eyebrow and bib to be significantly species-informative for the two species involved and thereby likely to be involved in species recognition. However, crown colour stood out as the most species-diagnostic trait. By using cline analysis to estimate widths and centres of clines and model the distribution of the different plumage traits, I detected different estimated selection pressures on the different plumage traits with crown colour being under strongest selection. These results suggest that plumage traits, at least crown colour, are under disruptive selection in the hybrid zone and thus, that it may contribute to behavioural isolation between the two taxa.

Differential selection pressure on male plumage traits

Hybrid zone clines should have narrow, sigmoid cline shapes with abrupt changes in parental character values in the centre when intermediate individuals are under selection. The narrow cline of crown colour stands out compared to the hybrid index and the other traits, hence implying selection on crown colour. The clines of cheek, eyebrow and bib were wider relative to crown colour and hybrid index suggesting weaker selection against intermediate types of these traits. This result is consistent with earlier observations of clinal variation in crown colour across the Alps hybrid zone (Hermansen et al., 2011) and was further confirmed by the strength of selection estimates which also singled out crown colour as being under strongest selection.

Steep character clines could arise even without selection acting on the trait in question if the two species involved have met recently, as neutral diffusion takes some time to flatten the cline (Endler, 1977; Hewitt, 1988 ; Barton & Gale, 1993). I consider this scenario highly unlikely for crown colour in the Alps hybrid zone. Using the cline width of 33 km from cline analysis and the conservative dispersal estimate of 2 km/gen (Anderson, 2006), the two species are estimated to have come in contact 43-44 generations ago. Assuming sparrow

generation time to be approximately two years (Jensen et al., 2008), the two species should have met in the Alps hybrid zone less than 88 years ago to achieve the observed crown colour cline width of 33 km. Older observations of hybrids between Italian and house sparrow prove contact between them for at least 125 years (Wallis, 1887), although this almost certainly is a very large underestimate as well. The sparrows most likely came in contact some thousands of years ago (Hermansen et al., 2011). If we assume 2000 years since contact, the crown colour cline should have been approximately 225 km wide today after one thousand generations of free diffusion. Hence, even when using highly conservative estimates of dispersal and time since contact, the most plausible explanation is that the crown colour cline is maintained narrow by selection against hybrids in the contact zone. However, the historical scenario leading to the formation of the sparrow hybrid zone in the Alps is not known. Trier et al. (2014) suggested a scenario in which genes incompatible between parental species have moved from where hybridization originally took place when the hybrid Italian sparrow was formed and only then came to rest at the current hybrid parent species range boundaries, including the Alps. Genetic variation was consistent with this scenario (Trier et al., 2014). If this is how the Alps hybrid zone was formed we would expect the movement of incompatibilities to have left behind broad clines in neutral alleles. The expected time since contact would then be overestimated, not underestimated. Hence, if the crown had been neutral under this scenario we would have expected it to possess a broad, shallow cline. Selection against intermediate crown colour is therefore still the most plausible explanation for the narrow crown colour cline.

Although I show differential selection pressures on different plumage traits across the Alps hybrid zone, one can only speculate the underlying reasons behind these patterns as I have not conducted mate choice or any other signalling experiment in this study. A plausible explanation for selection against individuals with intermediate crown colour is that they are unattractive to potential partners of either parental species (West-Eberhard, 1983; Wiernasz & Kingsolver, 1992). A possibility for the cline shapes and selection strength estimates of cheek, eyebrow and bib is weaker selection against these traits compared to crown colour across the Alps hybrid zone. However, the differential selection pressures may result from other forms of selection than selection against hybrids. The signals transmitted by plumage colouration may provide more information than being a conspecific or not. In birds, plumage

is also often used within species to signal, among other things, quality and social status (Dale, 2006; Hill, 2006). Given such selection pressures, females may not choose males according to species, potentially explaining the wider plumage clines in the Alps. The within-individual variation in cheek colouration (luminosity) contributed more to the trait being species diagnostic than that of the mean luminosity. That is, cheek colour apparently becomes less variable (i.e. lower contrast) to a greater degree than becoming lighter (i.e. whiter) when moving from the house sparrow to the Italian sparrow range. High contrast cheeks are likely to be partly white, alongside grey (pers. obs) allowing for varying levels of cheek contrast within species. The cline analysis showed a significant southward shift in cline centre of the cheek, suggesting movement of house sparrow cheek colour alleles into the Italian sparrow population. Cline shifts relative to the other coincident clines may result from selective advantage of the introgressing trait, potentially through environmental selection (Hewitt, 1988), or sexual selection by female choice or male-male competition. However, as grey and white cheek colour are unlikely to be involved in camouflage or other ecologically adaptive functions, sexual selection favouring the higher-contrast house sparrow cheeks seems like a more reasonable explanation. A similar scenario is observed in the well-studied manakin species system (Parsons et al., 1993). Two manakin species, the white-collared (*Manacus candei*) and the golden-collared (*M. vitellinus*) manakin, differ in several morphological characters, and cline analysis performed in their South American hybrid zone revealed shifts of plumage clines into to *candei* range, possibly a result of positive sexual selection for yellow plumage traits found in *vitellinus* (Parsons et al., 1993; Brumfield et al., 2001; Stein & Uy, 2006). Although sexual selection usually favours conspicuous colours and large contrasts (Andersson, 1994), such as the white cheek of an Italian sparrow against the black bib and the crown, one can argue that, given that different plumage traits have different cline positions and widths and hence may be under independent trait-specific selection, within-trait contrast may be more important than between-trait contrast for cheek.

Since ornamental plumage traits often are used by females as a criterion in mate choice (Hill, 2006), the wide cline for bib variation over the Alps hybrid zone might be explained by it playing a greater role in intraspecific competition for mates than in species recognition. Numerous studies have been conducted on the house sparrow to infer female mate choice

based on bib size (Møller, 1988; Kimball, 1996, 1997; Griffith et al., 1999; Mcgraw et al., 2003). However, the studies are contradicting (even within the same researcher: Kimball, 1996, 1997), with varying results across geographical areas and under different climate conditions. This may suggest that sexual selection on bib size is environmentally dependent as has been observed for ornamental traits in flycatchers (Robinson et al., 2012). Thus, the large bib variation across the Alps hybrid zone together with the observed larger Italian bib sizes further south in Italy (Töpfer, 2006; Hermansen et al., 2011) imply that the trait is more affected by intraspecific female mate choice or male-male competition than species recognition.

Females may choose mates after assessing their signals and recognizing them first as either con- or heterospecifics (species recognition) and second as of high or low quality (quality recognition) (Andersson, 1994; Dale, 2006). Different traits may be used in the two assessments. In summary, crown colour lacks variation within species and is under strong clinal selection in the hybrid zone, suggesting a major role in species recognition. This is consistent with the pattern observed when comparing cline widths and level of species-diagnostic character of the four plumage traits. The shifted cline in cheek colour implies asymmetric female preference if the more contrasting house sparrow cheek is favoured. The wide and poorly fitting clines for bib and eyebrow suggest either weak selection on these traits, or sexual selection that does not generate assortative mating via female choice, such as condition-dependent selection.

Methodological considerations

Quantification of colour patterns with digital photography provides data of better quality when colour standardization is done during the photographic session rather than later (this thesis, part II). Here, such corrections were done later. However, crown colour was measured using colour information per pixel, and gave reasonable results from high and low quality photos (with respect to focus and light variation). Cheek and eyebrow are more susceptible to the potential light variation remaining after this type of colour correction, as they are composed of neutral colours and quantified here by measuring the change in

luminosity. Nevertheless, others have managed to quantify plumage patterns without a colour standard (e.g. Dale, 2000; Fitze & Richner, 2002), and we were able to use the white paper background as a surrogate standard colour checker. The view of the bibs varied in different images, and hence I might to some extent have failed to capture the actual variation in bib size across the hybrid zone. A more objective quantification would be an improvement, alongside standardized bird position for bib photography (see Part II).

Geographical cline analysis as a method for identifying hybrid zone selection patterns in genomic and morphological traits is widely used in different taxa, including mice (Macholán et al., 2007 and Mullen & Hoekstra, 2008), grasshoppers (Bailey et al., 2012), salamanders (Alexandrino et al., 2005) and birds (Brumfield et al., 2001). Cline analysis is well-suited for this study as mate choice studies on this species complex have been difficult to accomplish (G-P Sætre pers. comm). Cline analysis visually displays the plumage distribution across the hybrid zone and allows for detecting and measuring the strength of selection acting on the characters investigated. On the other hand, the method cannot determine the type of selection pressures acting, and the mechanisms behind it (e. g. male-male interactions, female preferences, choice and pair formation) still remain unanswered.

When using cline theory to estimate cline structure and selection, there are biases to consider. The cline fitting procedure used here assumes that all populations have the same degree of trait variation; an assumption which is typical when fitting clines to quantitative traits. However, trait variances often increase in the cline centre (Barton & Gale 1993). Allowing the estimate of population trait standard deviation to follow a quadratic curve, peaking at the cline centre (Bailey et al., 2012; Shuker et al., 2005), would likely improve the fit of the model and narrow the confidence intervals for cline parameters. The large increase in crown colour variance in particular in the hybrid zone (Figure 7a) suggests that confidence intervals may be overestimated and that crown may in fact be significantly narrower than HI. Another bias is the dispersal estimate on which the equations for calculating both neutral diffusion and strength of selection across a hybrid zone are dependent (see equations 2 and 4). Accurate dispersal estimates are difficult to obtain due to limitations of the study area, which often fail to include long-distance migrations and historical events (Barton & Hewitt, 1985; Szymura & Barton, 1986; Barton & Gale, 1993). I used a direct average dispersal

estimate based on mark-recapture studies (from review in Anderson, 2006) which thus may underestimate dispersal. More precise dispersal estimates could be obtained using linkage disequilibrium (Barton & Gale, 1993; Alexandrino et al., 2005; Macholán et al., 2007; Gay et al., 2008) but these methods lie outside the scope of this thesis. Finally, the locations chosen as parental populations varied in sample size, and the house sparrow populations were somewhat close to the Alpine ridge (approximately 30-45 km) compared to the Italian (approximately 70-85 km), limiting how far north of the ridge the fitted cline could occur. However, as the hybrid zone centre is approximately 20 kilometres south of the Alpine ridge, the geographic bias is limited.

Premating barriers in a hybrid zone

Sexual signalling as reproductive barriers has been widely investigated in hybrid zones. These studies include hybrid zones between mice (*Mus musculus*/*domesticus,* Smadja et al., 2003), butterflies (*Heliconius cydno*/*melpomene*, Jiggins et al., 2001), toads (*Bombina bombina/variegata*, (Szymura & Barton, 1986), grasshoppers (several species of *Chorthippus,* Butlin et al., 1991; Bailey et al., 2012) and quite a few bird species, for instance flycatchers (*Ficedula hyboleuca/albicollis*, Sætre et al., 1997; Sæther et al., 2007), manakins (*Manacus vittelinus*/*candei*, Brumfield et al., 2001; McDonald et al., 2001), gulls (*Larus glaucescens*/*occidentalis,* Gay et al., 2008) and warblers (*Dendroica occidentalis*/*townsendi*, Rohwer et al., 2001).

In several of the species listed above, researchers have investigated the hybrid zone structure by comparing cline parameters (centres and widths) of sexual signalling traits with molecular markers and some also with other morphological traits, to investigate sexual signalling's role as a reproductive barrier. The mating call of *Bombina* toads differ, with *B. bombina* having longer calls than that of *B. variegata* (Szymura & Barton, 1986; Sanderson et al., 1992). Cline studies from the Polish hybrid zone revealed strongly concordant and coincident clines for the mating call, molecular markers (allozyme loci) and other morphological traits (Szymura & Barton, 1986). The same cline patterns were observed between the two grasshopper species *Chorthippus brunneus* and *C. jacobsi* in the Cantabrian Mountains in Spain. Bailey et al. (2012) compared male calling song, a differential signalling

trait among the two species, with a species-diagnostic, neutral morphological trait (stridulatory peg number), and found the two clines to be concordant and coincident. Hence, signalling traits in these studies are implied to be under the same magnitude of selection as all the other character(s) tested. In the two subspecies of *Mus musculus*, assortative preferences in males and females are affected by signals present in their urine (Smadja & Ganem, 2002). Cline comparisons between urine signal response and ten molecular markers (allozyme loci) in the Danish part of their hybrid zone revealed concordance and coincidence between eight molecular markers, but also a 10 km cline shift in male urine odour preference into *M. m. musculus* range (Raufaste et al., 2005; Ganem et al., 2008). A cline shift was also observed between *Larus* gulls when Gay et al. (2008) compared clines of seven molecular markers (six allozyme loci and one mitochondrial marker) with two morphology traits, namely plumage colour and colouration on bare parts of the birds, the latter suggested to be involved in assortative mating in seabirds. All clines were concordant, but the cline centre shift was observed in colouration in bare parts towards the *L. occidentalis* range. Some studies compare several plumage traits as done in this thesis, including the *Dendroica* warblers and the *Manacus* manakins. In the warblers, plumage traits are thought to signal aggressiveness between the two territorial species (Rohwer et al., 2001). Clines of seven plumage traits assumed to be involved in aggressive behaviour showed concordance and coincidence across their hybrid zone although significantly narrower than the cline of a molecular marker (mitochondrial) (Rohwer et al., 2001). In the manakins, Brumfield and collegues found concordant and coincident clines for six molecular markers (mitochondrial) and morphometric traits (combined to overall size). However, the lek-mating male manakin's collar and belly colour showed a large centre shift into the range of *M. candei*.

In summary, the overall pattern of sexual signals in these well-studied hybrid zones is being (1) concordant and coincident with other traits, thus implying genome-wide selection (*Bombina* and *Chorthippus*), (2) non-coincident centres explained by asymmetric mate preferences (the *Mus* mice) or sexual selection favouring one species (the *Dendroica* warblers, the *Larus* gulls and the *Manacus* manakins) or (3) concordant and coincident with other signalling traits (the *Dendroica* warblers and the *Manacus* manakins).

The plumage clines found in the Alps hybrid zone deviate from the patterns seen in the hybrid zones mentioned above: plumage traits are both non-concordant and non-coincident. What might explain the different type of variation in cline width and position between traits in the sparrow hybrid zone? One explanation may be the fact that the Alps hybrid zone is a contact zone between a hybrid species and one of its parents. The Italian sparrow is composed of genes from both parental species (Elgvin et al., 2011; Trier et al., 2014), and the Italian individuals inhabiting the Alps are already composed of about 75% house sparrow genes (HI is between 0.3 and 0 in all individuals used in this study, appendix 1). With this genetic similarity, there are likely to be fewer genes involved in reproductive isolation overall. This reduces the probability of physical linkage between genes. Reduced linkage reduces the chance of clines being pulled together and may therefore fail to cause genome-wide selection. Hence, the traits can evolve independently, potentially leading to both greater non-concordance and non-coincidence than seen in hybrid zones between non-hybrid species.

Future directions

Sympatric bimodality is suggested to be a way to define species (Mallet, 1995) and has also been suggested to involve prezygotic isolation (Jiggins & Mallet, 2000). Thus, increasing the sample sizes in hybrid populations across the Alps hybrid zone to allow tests of sympatric bimodal distributions in this species complex, would further contribute to the declaration of the Italian sparrow as a homoploid hybrid species.

Revealing the mechanisms behind the selection pressure in the hybrid zone could be achieved by finding ways to examine assortative mating directly in field populations. This could be done by setting up wild pedigreed populations in the hybrid zone, as has been done for a metapopulation of island-dwelling house sparrows in northern Norway (e. g. Jensen, et al., 2003). This would aid in the understanding of the relative importance of plumage variation across the Alps hybrid zone and further understanding of homoploid hybrid speciation.

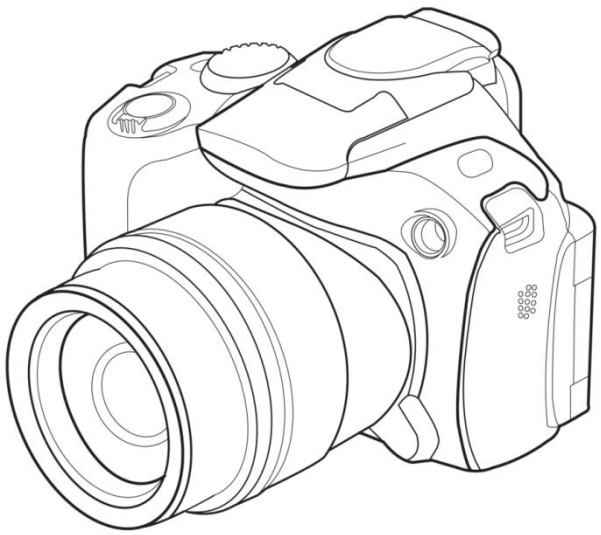
Conclusion

In this thesis, I have investigated variation in male plumage colour between the Italian sparrow and the house sparrow in the Alps hybrid zone. Using cline analysis, I found several lines of indirect evidence together implying selection against hybrids with intermediate crown colour. First, the crown colour exhibited a narrower cline than the hybrid index and the other plumage traits investigated here. Second, if crown colour were selectively neutral the width (of 33 km estimated here) would have require that the two species met only 43 generations ago which is highly unlikely. Finally, the calculations of selection strength highlighted crown colour as the trait under strongest selection. Therefore, I argue that these results provide evidence for crown colour being a premating reproductive barrier between the hybrid Italian sparrow and one of its parents, the house sparrow in the Alp hybrid zone. The other plumage traits, on the other hand, did not appear to be under similarly strong selection.

Sexual signals are important in species recognition, not only for the organisms themselves but also for taxonomists. The three sparrow species discussed here are primarily classified according to male plumage colouration. Hence, the existence of reproductive isolation, potentially caused by female preference for species-diagnostic male plumage, contributes to the declaration of Italian sparrows as a homoploid hybrid species.

Part II

A new method for quantifying colours of *Passer* sparrows using digital imaging in the field



Abstract

Accurate quantification of colour patterns is necessary to enable precise comparison and analysis to answer biological questions. As quantifying bird plumage colours from digital images turned out to be quite challenging in part I of this study, I also developed and improved methods for quantifying such variation when using digital imaging and image analysis. I invented a photo box with a manual flash system and standardized manual settings which constituted a standardized environment, and I implemented the use of a colour standard including a greyscale which allows for later colour correction. Tests comparing light variation between photos in the old and the new method showed that light variation was substantially lower in the new method. Furthermore, new analysis program utilizing the implemented colour standard and exploiting the data in a two-dimensional way, gave the best results in distinguishing colours. Together, the new method and the new analysis in a two-dimensional space yielded data of better quality. Hence, the data retrieved from the new standardized method will be of better quality and allow for more accurate image analysis.

Introduction

Investigation and quantification of colour patterns is necessary to enable comparison and analysis to answer many ecological questions. Many animals use visual signals such as distinct colour patterns as indicators of sex, breeding state, condition or rank (Hill & McGraw, 2006). These signals may be emitted in wavelengths visible to humans (400-700nm), within the ultraviolet spectrum (320-400 nm) or both (Cuthill, 2006). Through the years, different methods for colour quantification have been used, ranging from old-fashioned colour rankings through matching colour to official colour standard charts, to the modern techniques such as spectrophotometry and digital photography analysis, respectively (e. g. Baker & Parker, 1979; Bortolotti et al., 1996; Pryke et al., 2001; Stevens et al., 2007).

Reflectance spectrophotometry is probably the most widely used method for quantifying colours in birds. A spectrophotometer measures the amount of light a sample absorbs by passing a ray of light through the sample and then measuring the intensity of light reaching a detector (Endler, 1990). It allows for colour measurements outside the human visible spectrum, such as ultraviolet reflectance (UV). Even though spectrophotometry has these advantages, digital photography analysis might be more suitable and more practical in some situations. First, the majority of digital cameras have high spatial resolution – the resulting digital image is composed of a greater number of pixels than an image of lower spatial resolution. A digital camera with high spatial resolution allows for colour quantification from irregular surfaces, very small (e. g. through a microscope) or very large areas, and from some distance, which is not possible with spectrophotometry as the method needs to be in direct contact with the object measured and measures single points, one at a time (Montgomerie, 2006; Stevens et al., 2007). The spectral resolution, which describes a sensor's ability to define individual wavelengths, is lower in a digital camera compared to a spectrophotometer as the number of bands is much higher in a spectrophotometer (e.g. an RGB-imaging system such as a digital camera has three bands while a standard spectrophotometer has hundreds and often thousands). Further, the bands of the spectrophotometer also cover a wider part of the spectrum while an RGB-imaging system does not have any bands that cover the ultraviolet spectrum. However, it has been shown that the variation in plumage colours

retrieved from digital photography in a bird species (the *Quela quela*) was strongly correlated with the variation calculated from reflectance spectrophotometry (Dale, 2000). Moreover, even though a digital camera only shows the human-visible spectrum, this only constitutes a problem when the colours present in the study species are not covered by the cameras colour-system. If so, there is no major reason to use a spectrophotometer. Second, a decent digital camera is cheap and easy to use in comparison to a spectrophotometer. There is also a great amount of available programs to process the images, free or payable. Third, photography is field-friendly, as it is portable, non-invasive, enables rapid data collection, and the images can be stored for later analysis (Nguyen et al., 2007; Stevens et al., 2007). Thus, digital photography constitutes a valuable method for quantifying colours. The digital camera works by providing a reconstruction of the scene as an image: a matrix of hundreds of thousands pixels, each with its own colour value. The colour of a single pixel is measured using three colour sensors or channels. The most widely used colour model is the RGB model where each pixel is assigned a light intensity value (ranging from 0 to 255) of red, green and blue (RGB) (León et al., 2006). The correct reproduction of these RGB values provides the opportunity for colour quantification.

Digital photography as a tool in colour quantification is frequently used in food science (e.g. Yam & Papadakis, 2004) and also in ecological research, mainly in animal studies (e.g. Badyaev et al., 2001). As digital imaging only records colour variation within the human-visible spectrum, difficulties arise when investigating animals with the ability to see light with wavelengths outside of this, such as UV-A (320-400 nm). Birds, for example, have tetra chromatic vision which is different from the trichromatic vision of humans, mainly through the ability to see in UV (Kevan et al., 2001). This should be taken into account when studying animal colouration and behaviour.

A standard method is necessary for comparing different photos, and this could be achieved by using a colour standard and a controlled light environment, and standardised camera settings. Colour standards are calibration objects with known colour values, and they often consist of a grey scale from black to white (neutral colours) and a scale of primary colours (red, green and blue). After obtaining the images, calibration is necessary to account for effects of the ambient light and camera settings, and to correct for biases in the camera's

processing of the scene photographed (Stevens et al., 2009). By definition, there is a linear relationship when plotting the values of a grey scale against the reflectance values, meaning there should be a linear response to the changes in light levels of the image values recorded. Most digital cameras have a nonlinear response to this change in light levels, that is, a response deviating from the expected linearity, and therefore, calibration is needed to provide correct measurements of colour values (Stevens et al., 2007). By using a colour standard grey scale it is possible to correct any differences between the original known colour values and the captured values. The calibrations are performed with algorithms specific for the colour standard used. This improves the accuracy of the colour estimates as image specific deviations are corrected for.

Digital cameras are primarily made to facilitate the capture of a good-looking image. The automatic settings of a camera adjust the colours and contrasts according to the ambient environment to achieve this goal. In scientific research, where correct representation of the actual subject is what matters, usage of automatic settings should be avoided (Stevens et al., 2007). Calibrations and manual settings (assuring the settings are the same every single time), facilitate the later image processing and provide the level of accuracy in data measurements of colours necessary when the intention is to obtain data from a digital image useful for scientific investigation.

In this part of my thesis, I aim to make a new field-method for digital photography suitable for our purpose, which is photographing live *Passer* sparrows in the field, and overcoming earlier methodological problems, especially problems with illumination (see Part I).

The majority of *Passer* sparrows show plumage patterns consisting of brown, grey and black. Such colours are usually melanin-based pigmentation (Prota et al., 1995) and are within the human visible spectrum. This was confirmed by spectrophotometer tests on live *Passer* males in the field and on male *Passer* museum specimens from the collections at the Natural History Museum in Oslo by me and Prof. Arild Johnsen (Appendix 3). Some studies have reported UV colouration in *Passer domesticus* (see e. g. Lima et al., 2012) but when we re-analysed their raw data the spectra contained no peaks in the UV, and the UV reflectance reported in the study is most likely derived from the keratin structure of the feather (M Brydegaard, pers. comm.).

The sparrow plumage traits of interest are the crown, cheek, eyebrow, chest, bib, and back as they vary among different Passer species and can influence evolutionary processes. Several of these traits are larger areas where patterns and composition of colours are as important as the colour itself. Here, the colour difference between house sparrow (*Passer domesticus*) and Italian sparrow (*Passer italiae*) is used for testing the method. These species vary, among other things, in crown colour, possessing a crown of either grey (house sparrows) or brown (Italian sparrows, see Figure 1, Part I) feathers. As mention above, a spectrophotometer only samples single points and is less convenient when measuring patterns of larger areas, such as for example crown, for which intermediates in the Alps hybrid zone have mixed colours. In addition, the digital images can also be used in investigations of beak morphology and size measurements of plumage traits. Good quality spatial measurements from photos require a standardized set up. When holding the bird in the same few positions on a millimetre paper background, it is possible to perform better spatial measurements. Bib measurements, for example, could be standardized and measured using custom written programs (e. g. determine boundary of colour, as in Stevens et al., 2007). However, the focus in this study is to infer how well the new method with controlled illumination distinguishes grey from brown crowns.

I focused on the construction of a box as a controlled illumination environment for photography, and appropriate camera equipment, standardized camera settings and the use of a colour standard with supplementary colour correction program since these are essential for obtaining correct colour quantification with digital imaging (Villafuerte & Negro, 1998; Yam & Papadakis, 2004; Stevens et al., 2007, 2009). I also tested a new analysis program designed to improve colour measurements by better exploiting the data retrieved from RGB images to improve and facilitate the image analysis.

Materials and methods

Equipment

Colour standard. I used X-rite mini ColorChecker ®classic (5.7 x 8.7 cm, manufactured by X-rite Photo) as our colour standard. The ColorChecker consists of 24 coloured squares in four rows, representing a grey-scale, primary colours and natural colours (Figure 10). This colour standard has a colour correction package, "Chromatic Spatial Variance Tool Box" (Brydegaard, 2013) based on the method presented in Brydegaard et al. (2012) which performs the colour calibrations necessary for our images. This package linearizes the colour values with the Xrite greyscale and also excludes depleted or saturated pixels (a phenomenon called clipping) by removing pixels with RGB values below 1% and over 99%. The package is written in Matlab (Mathworks Inc.) and the colour corrections were done using this platform.

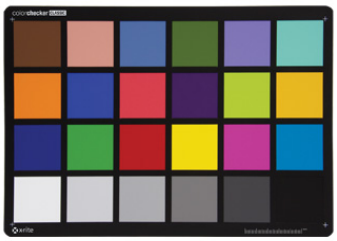


Figure 10: The colour standard used.

Camera equipment and camera settings. I used the same Nikon camera as in previous field seasons since our camera has the ability to save in RAW file formats, has manual white balance, and manual exposure control - three of the most important characteristics when choosing a digital camera for usage in colour research (Stevens et al., 2007). RAW files are the raw data of the image where no information is lost in compression into a smaller image (Yam & Papadakis, 2004), and thus provides more accurate colour data. White balancing assures that white objects in real life also are reproduced white in the photo, and allows for standard white balancing based on the specific light source used, in our case a flash. Exposure control consists of aperture, shutter speed and ISO speed, all controlling how light or dark the image will appear. The aperture (f-values, ranging from f/1-f/32) controls the area over which light can pass through the camera lens, and a high aperture value represents a smaller/narrow opening of the lens and a shallow depth of field. I chose f/8, a narrow to intermediate aperture, suitable for our standard positioned, and close-up photography (G Holm, pers. comm.). The shutter speed controls the duration of the

exposure (ranges from 1-1/4000 sec), that is, how much light is permitted to enter the camera, and it mainly controls the sharpness of the image. As our camera is attached in a fixed position, I chose the default setting of 1/125, which after testing gave sharp and clear images. The ISO speed (ISO 100-800) determines how sensitive the camera is to incoming light. Low ISO values (low sensitivity to incoming light) are desirable since they decrease image noise, which lowers the quality of the image. Again, the default setting, ISO 100, was chosen. All settings were set manually and were kept as the standard. A summary of the equipment specifications is listed in table 4.

I used a wide-angle lens with a lens-specific polarization filter attached. The wide-angle lens, as the name implies, has a larger angle of view than the standard lens of the camera, and the polarization filter is used to remove glossy reflections and improve contrasts. Glossy reflectance appears as white or grey areas in the resulting image giving an incorrect reproduction of the area photographed.

I used a high quality flash with wireless flash-trigger system. The wireless flash-trigger system allows the flash to be separated from the camera which was necessary for the camera setup to achieve the proper light conditions.

Table 4 Camera specifications used in the new method

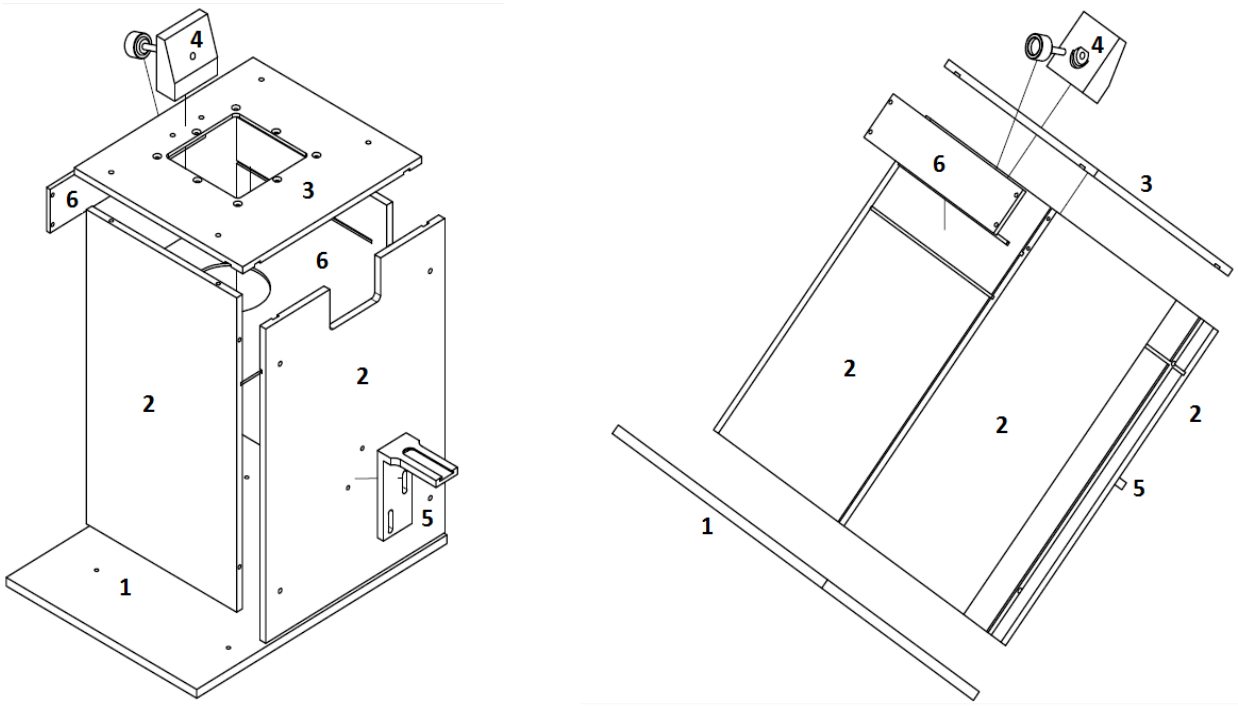
Camera	Nikon 5100
Image quality	RAW
Image size	Large
Exposure mode	Manual
White balance	Flash
Sensitivity	ISO 100
Release mode	S
Focus Mode	AF-S
AF-area mode	Single-point AF
Metering mode	Matrix metering
Active D-lighting	OFF
Auto bracketing	OFF
Picture control	Standard
Exposure compensation	0.0
Flash compensation	0.0
Flash mode	ϟ
Aperture	F8
Exposure time	1/125
Lens	Sigma 10-20mm F4-5.6 EX DC HSM
Focal length	10mm
Polarisation filter	Sigma DG Wide MC CPL Filter 77mm Circular polarisation filter, narrow type
Flash	Nikon Speedlight SB-700 TTL-Auto flash
Flash strength	1/8
Wireless flash-trigger system	Phottix Strato II Radio Trigger Nikon

Photo box

A photo box was constructed to accomplish a controlled environment with standardised lighting suitable for making colour measurements with digital photography. We wanted a box appropriate for our purpose, but not too complex or expensive, to facilitate usage in the field.

The box (Figure 11 and Appendix 4) consists of nine parts: a base plate, three walls, a lid and a camera holder, all made out of robust PVC (polyvinyl chloride, plastic), an aluminium flash-holder and two parts constituting the light compartment, and it can easily be dismantled and re-assembled for ease of transport. The size of the box is 36x20x20 cm. All three walls are covered with polished, stainless steel, (AISI 316, 1 mm), to function as mirrors. A test using standard mirrors made of 5 mm glass and aluminium sheet gave "ghost images" (several reflections as a halo around the specimen, due to the thick glass plates), therefore, we used polished metal plates which gave better result. Mirrors on all walls provide a better view of each specimen photographed, and all angles will appear in one photograph since we are using a wide-angle lens. The lid has a hole for the lens, bordered with black rubber to isolate from light admission and release. The box allows for a set-up with a constant camera distance.

The upper part of the box holds a 5x20x20 cm light diffuser compartment. It is made out of a thinner milky-white Plexiglas plate (PMMA Oroglas Harmony 800) at the bottom which allows for light emission, and thicker opaque plexi plates (Vekaplan SF) on all four sides, which totally isolate the compartment. The metal flash holder is made to assure that the flash protrudes into the light compartment. The light diffuser compartment functions to spread and diffuse the strong directional flash. I performed a flash test to determine the flash strength to use. A stuffed bird specimen was placed in the assembled box and all possible flash strengths were tested. I chose the strength that provided the clearest and most illuminated image and kept this setting as a standard (1/8).



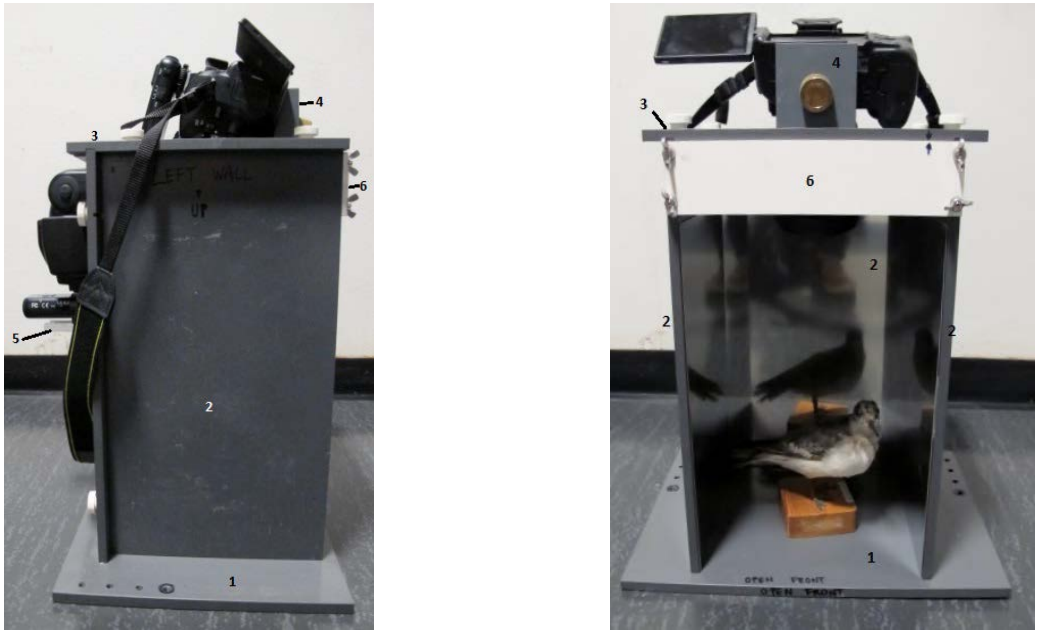


Figure 11 Above: Technical drawing of the photo box, without camera equipment. Below: Photo box with camera equipment fully set up. The different parts are shown with numbers in both figures: Base plate (1), three walls (2), lid (3) camera holder (4), aluminium flash-holder (5) and two parts constituting the light compartment (6).

Since we are working with live birds, the front of the box has to be open to enable hands on the specimens to avoid injuries to the birds. This requires the presence of the colour standard in each photo to later correct for possible light difference between the images (Stevens et al., 2007; Bergman & Beehner, 2008), even though we use standard lighting and settings. As our colour standard is of small size and our box large in comparison, this was not a problem. The colour standard should nevertheless be close to the specimen (Stevens et al., 2007; Bergman & Beehner, 2008). The base is covered with millimetre paper for potential later scaling, and the same type of millimetre paper was used in every photo to facilitate processing of the picture with regards to spatial measurements.

Testing the new methods in the field

The new methods for digital field photography were tested during the field season of 2013 which took place in Sicily, Crete, Corsica and Sardinia. A box manual for assembly (Appendix 5) and a field protocol (Appendix 6) were developed to facilitate usage of the new method and to assure that the correct settings of the equipment were used. This also assures the usage of the same settings during future fieldwork and that the settings used will be consistent across participants. All photos were taken with the box placed inside a car or in the shade to avoid light variation as much as possible.

Analysis

All statistical analyses were done using the statistical program R (R Core Team, 2013).

Effect of box and new settings

To assess the effect of the new box and new settings on RAW data, I investigated if there were any differences in the variation in illumination between the images taken with the old setup and the variation in illumination between the photos taken with the new box and setup.

I randomly selected 25 old and 25 new photos in RAW format. All photos were from the same angle (profile), and the old photos that were removed due to low quality for part I of this thesis were not used. Light measurements were recorded as the mean of luminosity, using the histogram function in Adobe Photoshop. Seven selections of the white background paper were done per photo, three selections around the bird and one selection in each corner of the image.

A Bartlett's test was performed to investigate whether the light variation between images taken with the old settings and the images taken with the new settings and the box, differed significantly, and the variation was displayed in a box plot.

Effect of new method including colour correction and effect of new analysis

To assess the effect of the new method, which includes colour correction by utilizing a colour standard and a concomitant linearization algorithm, I investigated how well the old and the new method distinguished and classified totally brown crowns from mixed crowns containing both grey and brown (further referred to as grey).

The old method uses the Matlab-script from part I of this thesis, extracting the R, G and B values per pixel and, in addition, provides the mean and standard deviation of those values for the selected plumage area. The new method uses a combination of two Matlab scripts from the Chromatic Spatial Variance Toolbox. The first script (called "histBatch") applies the colour correction in the form of a linearization based on the grey scale of the colour standard included in all images retrieved from the new photography method. The second script applies a principal component analysis (PCA) of the per pixel based colour data organized in either a one-, two- or three-dimensional space (called either "SVD1D", "SVD2D" and "SVD3D", respectively). Appendix 7 contains further explanation of the Chromatic Spatial Variance Toolbox and figure 4 from Brydegaard et al. (2012) visually showing the 1D, 2D and 3D distributions of the spectral bands (published with the authors approval). The eigenvalues (1D), eigenvectors (2D) and eigenplanes (3D) from the PCA are then used to determine how many of the Principal Components (PCs) are needed to accurately represent the data set. The break point is determined by visual inspection of a plot of successive

eigenvalues, eigenvectors and eigenplanes and determined to be at the point after which the values drop less rapidly. In this comparison, I used two different PCA analyses, the one-dimensional and the two-dimensional analysis (M Brydegaard, pers. comm).

To test if the method I applied in part I and the new method presented here are detecting the biological variation we are interested in, I performed three tests which I later compared: (1) a test of how well the old method distinguishes the crown colours, (2) a test of how well the new method which includes colour correction (corrections by the "histBatch" script) distinguishes brown and grey , and (3) a test of how well the new method including colour correction and PCA-based analysis (SVD) of the colour-corrected data distinguishes the crown types. Canonical variate analysis (CVA) was used to analyse how well the different methods distinguish between the two groups (brown and grey), and the Mahalanobis distances between groups and associated p-values were used as a measure of how well the methods could differentiate the groups.

The sample photos used in all tests were the first eleven photos of totally brown crowns and the first eleven of mixed crowns (including both grey and brown colour) from the field season where we used the new methods including box, new settings and the use of a colour standard (spring 2013). For the first test, mean and standard deviation of R, G and B respectively, were used in the analyses. The second test was performed to evaluate the colour correction script (histBatch) by itself, and RGB pixel values per individual were extracted in Matlab using the new script. The pixel values were then used in calculations of the RGB mean and standard deviation, which again were used in comparison analysis (the CVA). The third test was performed to evaluate the new script (histBatch) and the new analysis method (SVD scripts) together, and the output factor loadings provided by the automatic PCA were used in the comparison analysis. The number of principal components used in the CVA was chosen after inspection of the decay of the eigenvalues from the graph supplied from the SVD analyses in Matlab. Twelve PCs and fourteen PCs were used for SVD1D and SVD2D, respectively (Figure 12).

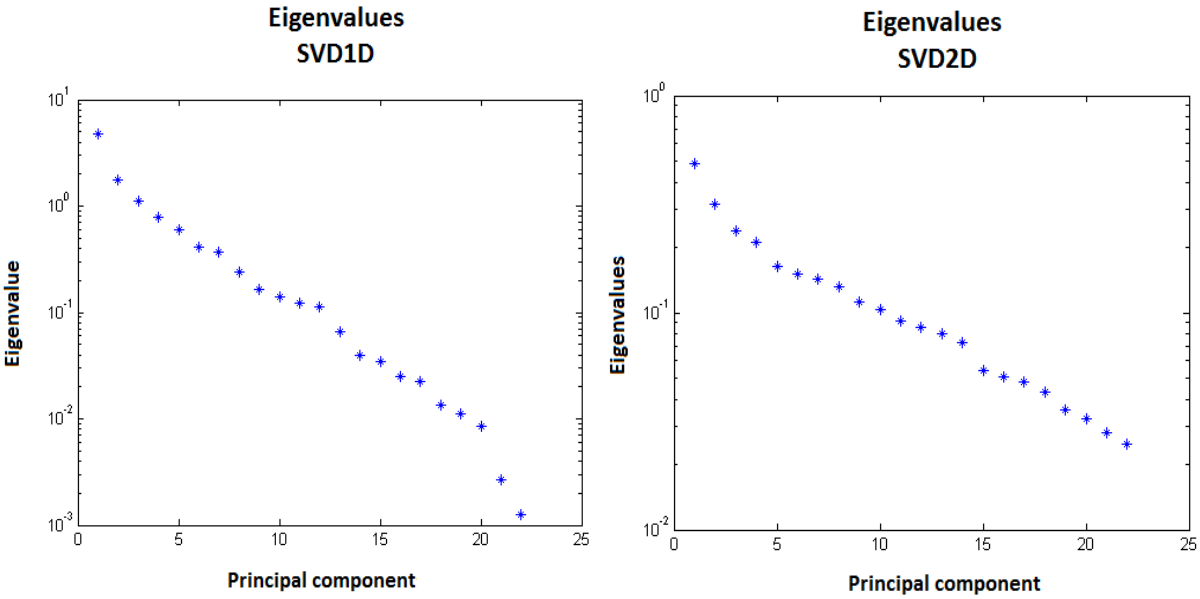


Figure 12 Plots of the decay of the eigenvalues retrieved from both principal component analyses (PCA). The plots were used to decide how many dimensions were needed to explain the variation in this dataset, that is, how many principal components to use in PCA to distinguish brown and grey crown colour. The left graph shows the eigenvalues from the PCA in one dimension (SVD1D) where 12 principal components were used, and the right graph shows the eigenvalues from the PCA in two dimensions (SVD2D) where 14 principal components were used

Results

Effect of box and new settings

The variation in light difference (mean luminosity) between photos was significantly lower using the new compared to the old method (Figure 13, p=7.417e-10).

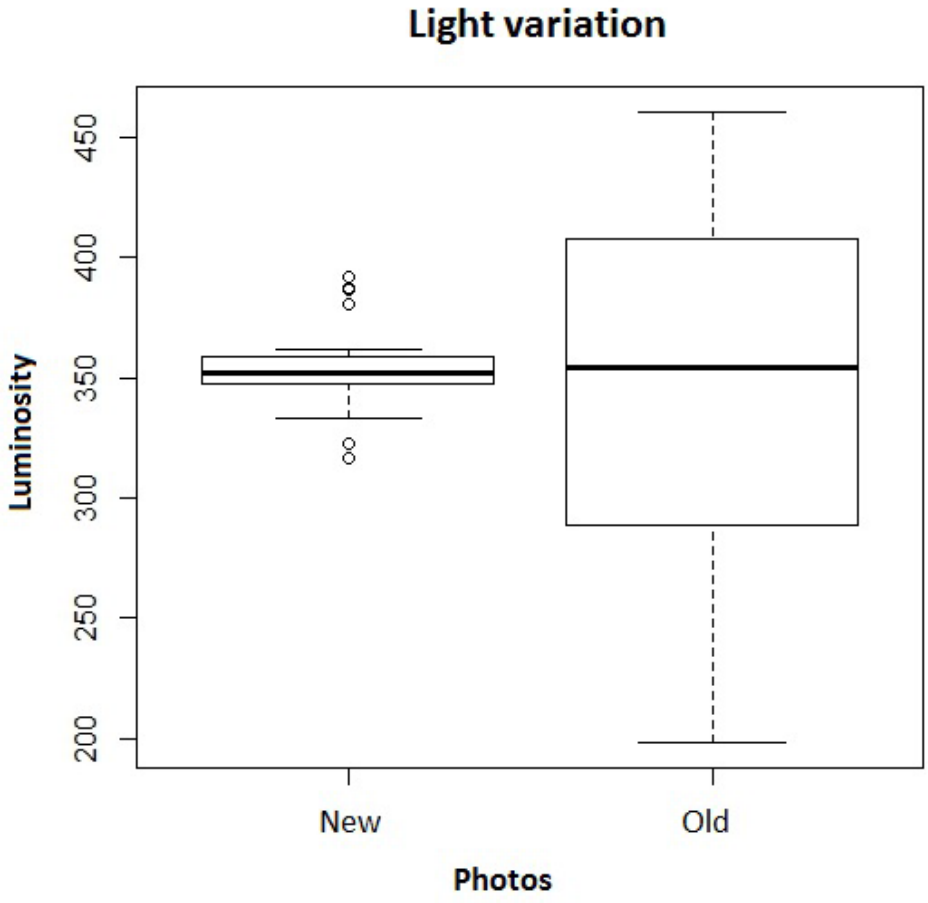


Figure 13 Boxplots of the light variation between photos, showing significant difference in mean luminosity between photos using the old and new method. The x- axis shows the group which was tested, either from the new or the old method. The left box shows the light variation between photos when using the new method, and the right box shows light variation retrieved from the old method. The y-axis is the mean luminosity which is used as a measurement of illumination. Each box shows the range in luminosity with the transversal line representing the middle value (median) of the luminosity datasets. The spread of the luminosity values are represented by the box where the lower and upper lines are the 25th quartile (Q1) and the 75th quartile (Q3), respectively, with the "whiskers" (dotted lines) as the luminosity values greater or less than Q1 or Q3. The maximum and minimum luminosity values are represented by the upper and lower horizontal lines, and the dots are outliers – the observations distant to the rest of the dataset.

Effect of new method including colour correction and new analysis

All methods distinguish the sample selection significantly (Table 5, $p<0.001$ and Figure 14). Somewhat surprisingly, the second test, using the mean and standard deviation calculated from RGB pixel values retrieved from the new script where the grey-scale based linearization is implemented, Mahalanobis distances were lower (MahaDist=5.20) than when using the old method. However, when including the new analysis program (SVD), the new method better distinguishes brown and mixed crowns (Table 5, MahaDist=6.76 and 7.78).

Table 5 An overview of results of the four different tests done to infer how well the old and the new method distinguished and classified brown and grey crowns. The first test was performed using the old method (1), the second test was done using the new method which includes colour correction (2), and the last test was performed using the new method with colour correction together with the new analysis methods SVD1D or SVD2D (3). All tests were significant with p-values < 0.001. Of all tests, "New script + SVD2D" distinguished the crown colours best with the greatest mahalanobis distance.

	Old script (1)	**New script alone (2)**	**New script + SVD1D (3)**	**New script + SVD2D (3)**
Variables	Mean & SD	Mean & SD	12 PC	14 PC
Mahalanobis				
Distance	6.33	5.20	6.76	7.788
Probability	0.001	0.001	0.001	0.001

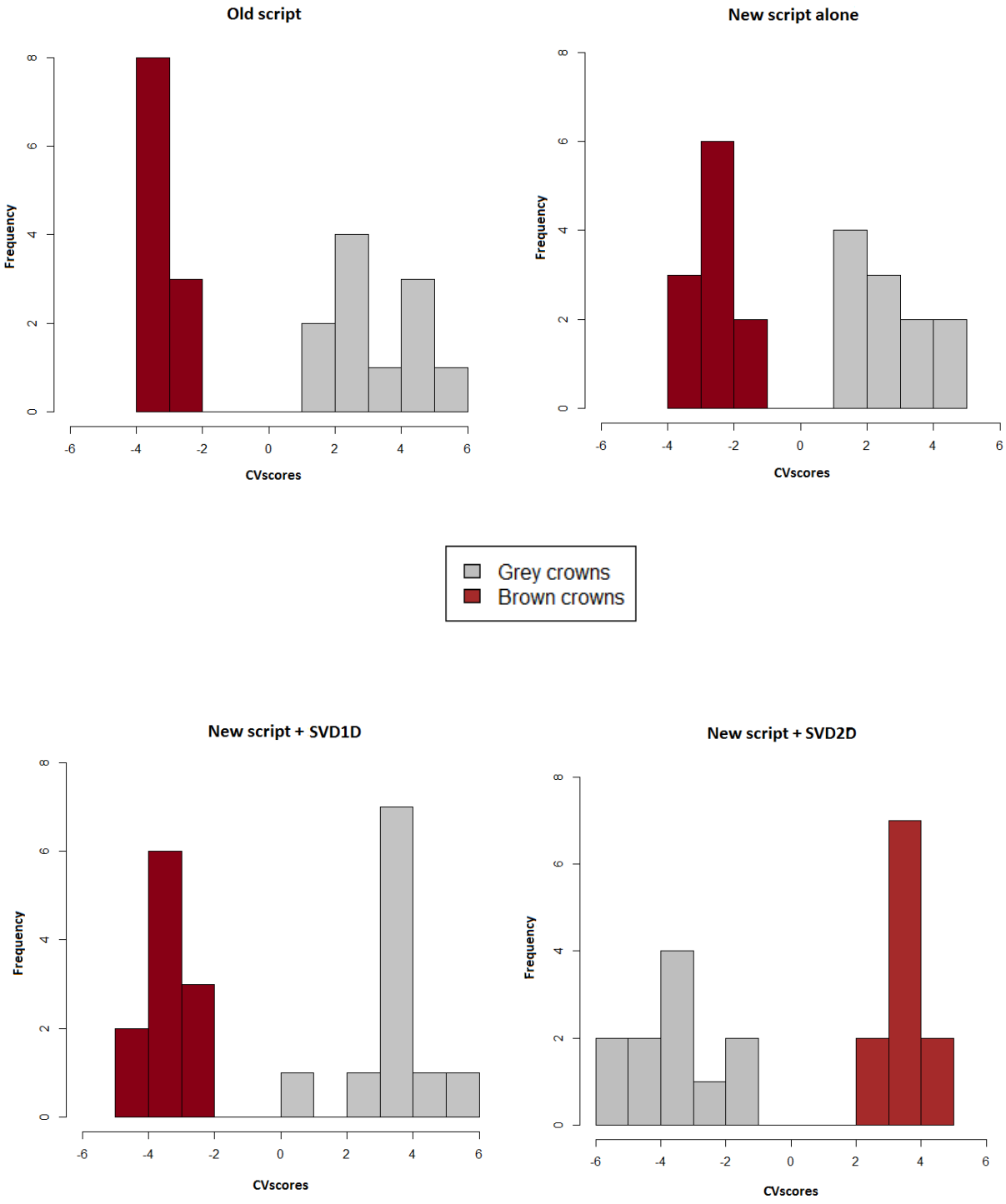


Figure 14 Histograms of all tests performed to investigate how well each test distinguished brown and mixed crowns of *Passer* sparrows showing full separation between brown and mixed crown colour in all cases. The x-axis shows the CV scores retrieved from the canonical variate analysis (CVA) used to distinguishing the crown colours, ranging from -6 to 6. The y-axis shows the frequency of individual crown falling in either of the two colour groups.

Discussion

By implementing a standardized method for digital photography, with emphasis on a standardized environment and the possibility of colour correction (linearization) during later processing, image quality can be significantly improved. In this project I have developed a photo box with a manual flash system and standardized manual settings which constitute a standardized environment. In addition I have implemented the use of a colour standard including a greyscale which allows for later colour correction (e. g. linearization). Hence, the data retrieved from the new standardized method will be of better quality and allow for more accurate image analysis.

Differences in light variation between old and new method

Standardized methods for measuring colour with digital photography are essential to obtain precise measurements. Still, as there is no overall standard method of doing these measurements (Yam & Papadakis, 2004), low quality data could be the result of poor standardization methods. One of our main difficulties regarding the old methods was variation in luminosity. For non-field photography this can easily be solved by taking photographs inside dark rooms, within a Light Box (Lehnert et al., 2008) or with a steady light system (e.g. a Repro light unit as in Alonso-Alvarez et al., 2004). Our new method deals with this problem by creating more constant illumination with a portable photo box including a flash system providing standard lighting. Portable boxes are field-friendly, ensure standard illumination, but are often used for immobile study objects such as in food science (e. g. Luzuriaga et al., 1997), bird eggs (e. g. Wegryn & Leniowski, 2011) or feathers (e. g. Jones et al., 2010). Since our study species are live birds which require a constant grip to be held in place, the box is designed with one open side to allow for hands. Nevertheless, the analysis confirmed a substantial improvement from the old methods (Figure 13).

Evaluation of colour correction and analyses of colour data

Studies quantifying animal colouration using digital photography often uses colour standards and a program especially made for that specific standard (e. g. Bergman & Beehner, 2008; Runemark et al., 2010; Runemark & Svensson, 2012). Here, an X-rite colour checker and the Chromatic Variance Toolbox were used.

I expected to see an improvement in distinguishing brown from grey colour when using the new program. Further, I expected the improvement (measured in significance and Mahalanobis distances) to increase with tests in this order: "Old", "New alone", "SVD1D", "SVD2D". All tests distinguished the colours significantly, and this shows that the method used in part I of this thesis also is useful and reliable. However, regarding the expected order of improvement, the old script seems to distinguish the colour slightly better than the new script alone (in both cases using mean and standard deviation of R, G and B of the selected whole crown area), but the two SVDs still distinguish better than "Old" and "New alone", and increase with dimension (1D and 2D). The latter is consistent with the expectation of SVD to increase prediction performance in comparisons with averaged values of RGB, and increasing with increased dimensionality which allows for greater utilization of the dataset (Brydegaard et al., 2012). A possible reason is the small test sample size. The sample size was small due to lack of birds with grey crowns from the fieldwork in 2013 where the new method was used. This might have contributed to the apparent lack of improvement from "Old" to "New alone" since Brydegaard et al. (2012) mention that small sample size in one of the colour groups tested in their case study gave a poorer result and since linearization should improve the colours (Stevens et al., 2007, 2009 ; Brydegaard et al., 2012). On the other hand, a small sample size should affect the old script just as much as the new one with colour correction included, especially since the same photos were used for both scripts. All the images used in testing the new method were off course photographed with the new equipment. As mention above, the photo box and its light system significantly reduced the light variation across images. Thus, another potential explanation could be that assuring a standard light environment (the photo box and the flash system) was good enough for distinguishing the colours of interest in this particular case.

Conclusions

We were in need of a method to acquire good, standardized bird photos in the field that could be utilized during different field seasons by different people, and that was easy to use. Especially for birds, a new method was created including standardized lighting with a light diffuser, a colour standard, standard camera settings (including manual white balance and exposure control), and using a set-up with constant camera distance and standard positions of birds on a scaled background. The box was robust, easy to travel with and simple to put together. Tests comparing light variation between photos using the old and the new field setup showed that light variation was substantially lower using the new setup. In addition, using the new analysis program performing colour correction based on a colour standard and using an analysis method exploiting the data in a two-dimensional way gave the best results in distinguishing colours. The new method together with the new analysis in a two-dimensional colour space yield data of better quality. This method works well for our study species, as sparrows do not exhibit UV-coloured plumage. If extended to species with UV colouration, the species should also be measured using spectrophotometry to capture all biologically relevant colour information.

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Appendices

Appendix 1

Supplementary table 1: Overview of location, plumage type based on visual investigation of morphology, mean population hybrid index, coordinates (in decimal degrees) and distance from alpine ridge and sample size from north to south in the Alp hybrid zone where the hybrid species *Passer italiae* meet and hybridize with one of its parental species, *Passer domesticus*.

Number	Site	Plumage type	Hybrid index (pop. mean)	Ridge distance (km)	Sample *N*	Latitude	Longitude
1	Kiesen	*P. domesticus*	0,020	43,76	7	46,804	7,575
2	Thusis	*P. domesticus*	0,070	35,17	4	46,696	9,441
3	Seedorf	*P. domesticus*	0,054	33,92	2	46,880	8,620
4	Ilanz	*P. domesticus*	0	29,12	1	46,766	9,209
5	Juf	*P. domesticus*	0,008	4,5	5	46,446	9,579
6	Bondo	Hybrid	0,098	-4,94	2	46,335	9,553
7	Olivone	Hybrid	0,086	-11	5	46,528	8,934
8	Lirone	Hybrid	0,138	-14,98	1	46,368	9,361
9	Castro	Hybrid	0,136	-16,38	5	46,477	8,933
10	Lostallo North	Hybrid	0,130	-19,53	1	46,322	9,201
11	Campagnola	Hybrid	0,133	-22,79	3	46,277	9,390
12	Piani Di Verdabbio	Hybrid	0,128	-22,87	5	46,283	9,174
13	Malvaglia	Hybrid	0,204	-24,65	1	46,405	8,979
14	Roveredo	Hybrid	0,151	-26,49	5	46,242	9,137
15	Personico	Hybrid	0,155	-27,8	4	46,374	8,911
16	Lumino	Hybrid	0,133	-29,12	1	46,229	9,067
17	Claro	Hybrid	0,153	-29,62	6	46,249	9,017
18	Iragna	Hybrid	0,155	-32,96	8	46,327	8,973
19	Lodano	Hybrid	0,189	-34,96	1	46,263	8,686
20	Aurigeno	Hybrid	0,129	-37,8	15	46,236	8,713
21	Gravedona	*P. italiae*	0,153	-39,97	6	46,130	9,289
22	Locarno Camping	*P. italiae*	0,156	-45,13	3	46,155	8,800
23	Porlezza	*P. italiae*	0,204	-49,39	5	46,034	9,135
24	Locarno Piano	*P. italiae*	0,188	-49,69	8	46,169	8,893
25	Bellinzona South	*P. italiae*	0,207	-50,21	6	46,174	8,982
26	Maccagno	*P. italiae*	0,244	-58,9	2	46,042	8,737
27	Mandello Del Lario	*P. italiae*	0,112	-65,69	2	45,913	9,316
28	Valbrona	*P. italiae*	0,178	-65,72	1	45,869	9,286
29	LeccoS	*P. italiae*	0,256	-66,09	3	45,783	9,424
30	Savosa	*P. italiae*	0,143	-67,09	3	46,020	8,942
31	Castel Veccana	*P. italiae*	0,233	-70,5	4	45,949	8,678
32	Brusimpiano	*P. italiae*	0,260	-73,54	2	45,946	8,890
33	Melide	*P. italiae*	0,138	-74,47	3	45,952	8,950
34	Sciranna	*P. italiae*	0,189	-84,21	5	45,802	8,779
35	Coldrerio	*P. italiae*	0,209	-85,61	3	45,852	8,986

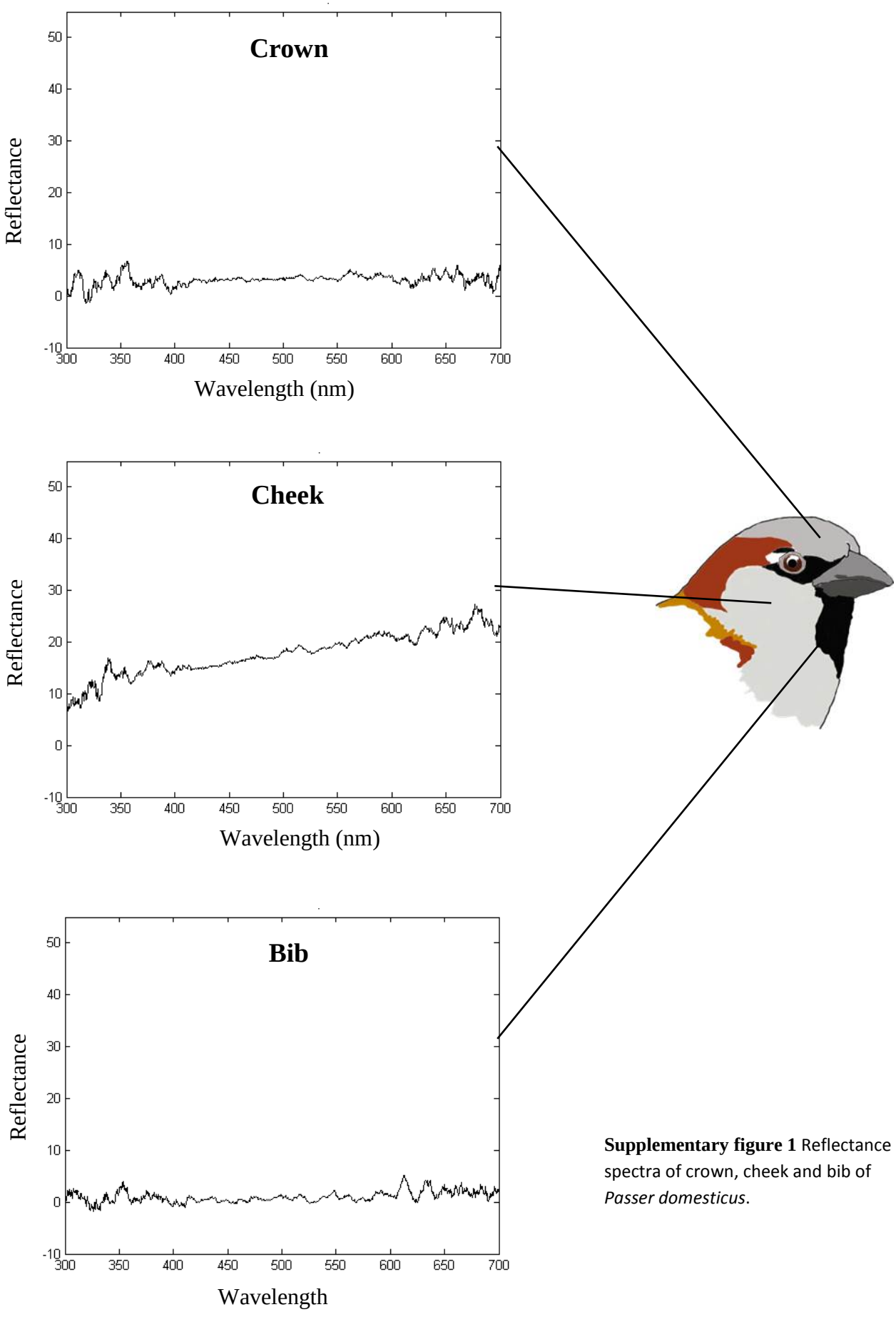
Appendix 2

Supplementary table 2: The 81 SNP's (Single Nuclear Polymorphisms) (see Trier et al., 2014) used to create a molecular hybrid index with purpose to be the genome-wide average which was compared with other plumage traits to infer if they were under selection.

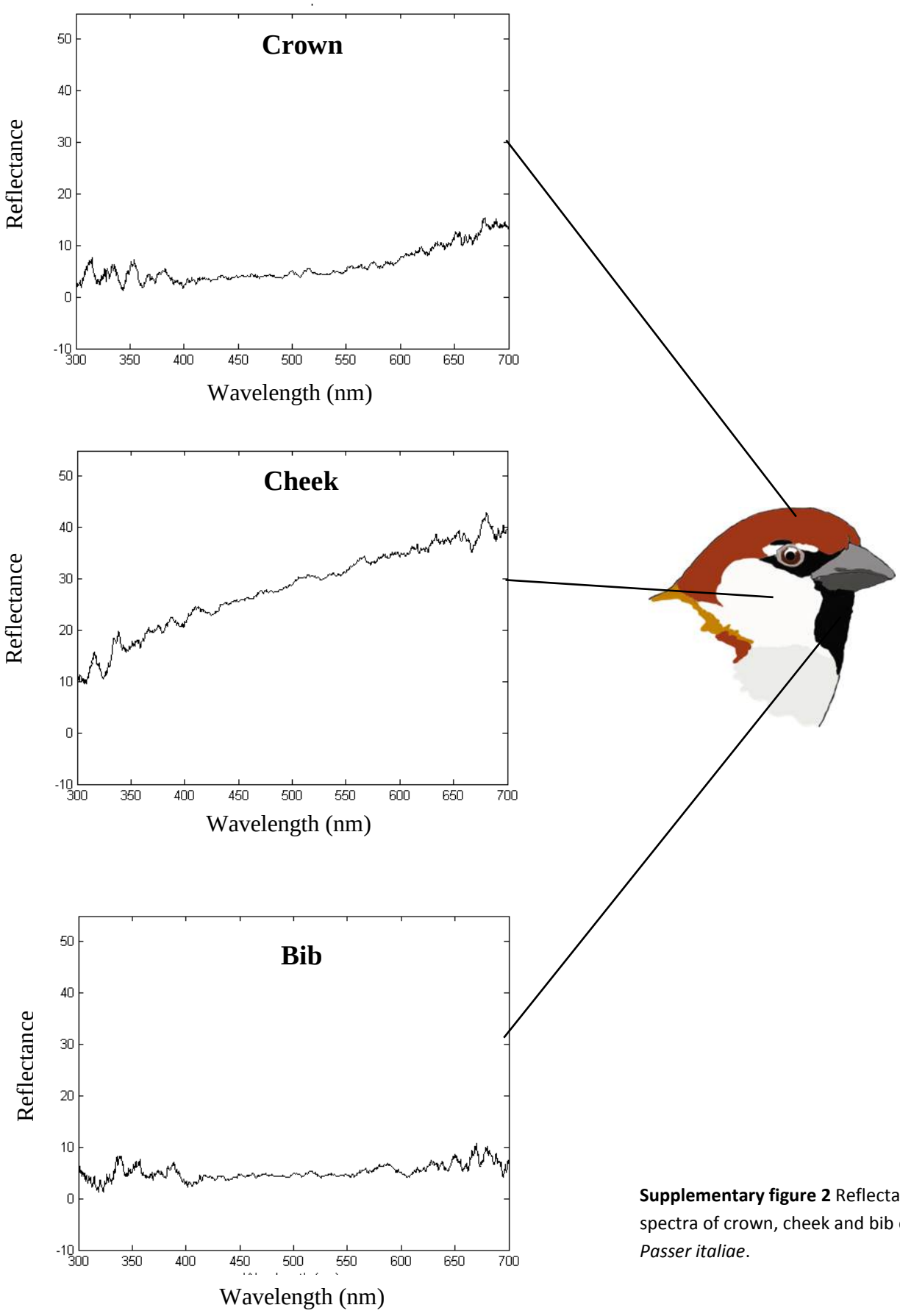
Loci		
ND2_2	A2ML1_6	SMARCA1
LNPEP	GSTK1	RPS4_1
CLTA	STAUR	TFIID1
NFIL3	COG5	RASGRP1
SECISBP2	SLC38A2	HECTD1
ZCCHC6	VWF_2	ARHGAP5
CETN3	DYRK4	SMEK1
ZFAND5	LANCL2	BTBD7
APC_2	HypC2	USP47
APC_6	CDC2l5_2	CCAR1
REEP5	PTPRM	HypPC6
SNX2	RB1CC1	GSTO2
CHD1Z_1	CRLS1	OSBPL6
HSDL2_1	WDR92	LPPR1
ACO1	PPP3R1	EPS15
MCCC2	MIA3	C8B
GTF2H2_2	RRP15	PRRC2C_2
TJP2_1	GNPAT_3	ILKAP
ADFP	Vps20_1	KIAA1370
MAP1B	SLC22A2_2	COPS2
CNKSR2	PHF3	ETFA_1
MYCBP2	YWHAQ	MTMR14
NDFIP2_1	G3BP2	EGR1
CHORDC1	ANKRD17_2	PITPNC1
TMEM135	FAM13A1	PECAM1
A2M_1	EFHA2	CLIP2
A2ML1_2	STIM2	NCOA3

Appendix 3

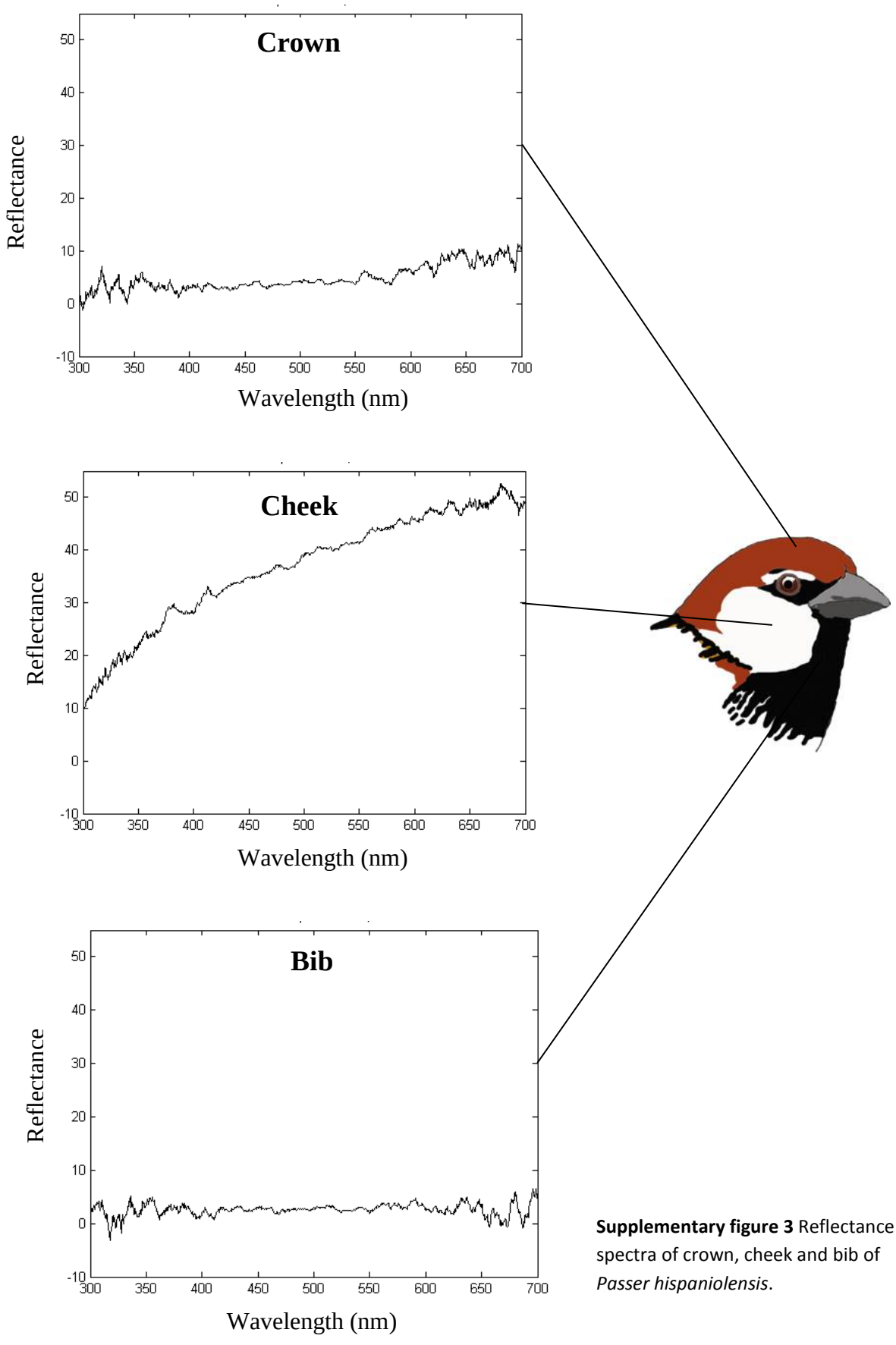
To confirm the lack of UV colouration in the plumage of *Passer* sparrows, spectrophotometry was performed on museum specimens from the Natural History Museum in Oslo with help from Professor Arild Johnsen. Reflectance was measured with an USB 2000 spectrometer (Ocean Optics, Eerbek, Netherlands) connected by a bifurcated fiberoptic probe to a Xenon (PX-2) pulsed light source. Reflectance was calculated relative to a WS-1 white standard (Ocean Optics). Crown, cheek, eyebrow, back and bib was measured three times per species, and the measurements were done on one specimen from each species (*P. domesticus*, *P. italiae*, *P. hispaniolensis*). UV reflectance would reveal itself by showing a peak in the spectra plots within the range of UV wavelengths (320-400 nm). None of the plumage traits in any of the species showed any UV reflectance (see Supplementary figures 1-3 for the spectra of crown, cheek and eyebrow), thus settling that there is no UV colour in the plumage traits of the *Passer* sparrows.



Supplementary figure 1 Reflectance spectra of crown, cheek and bib of *Passer domesticus*.



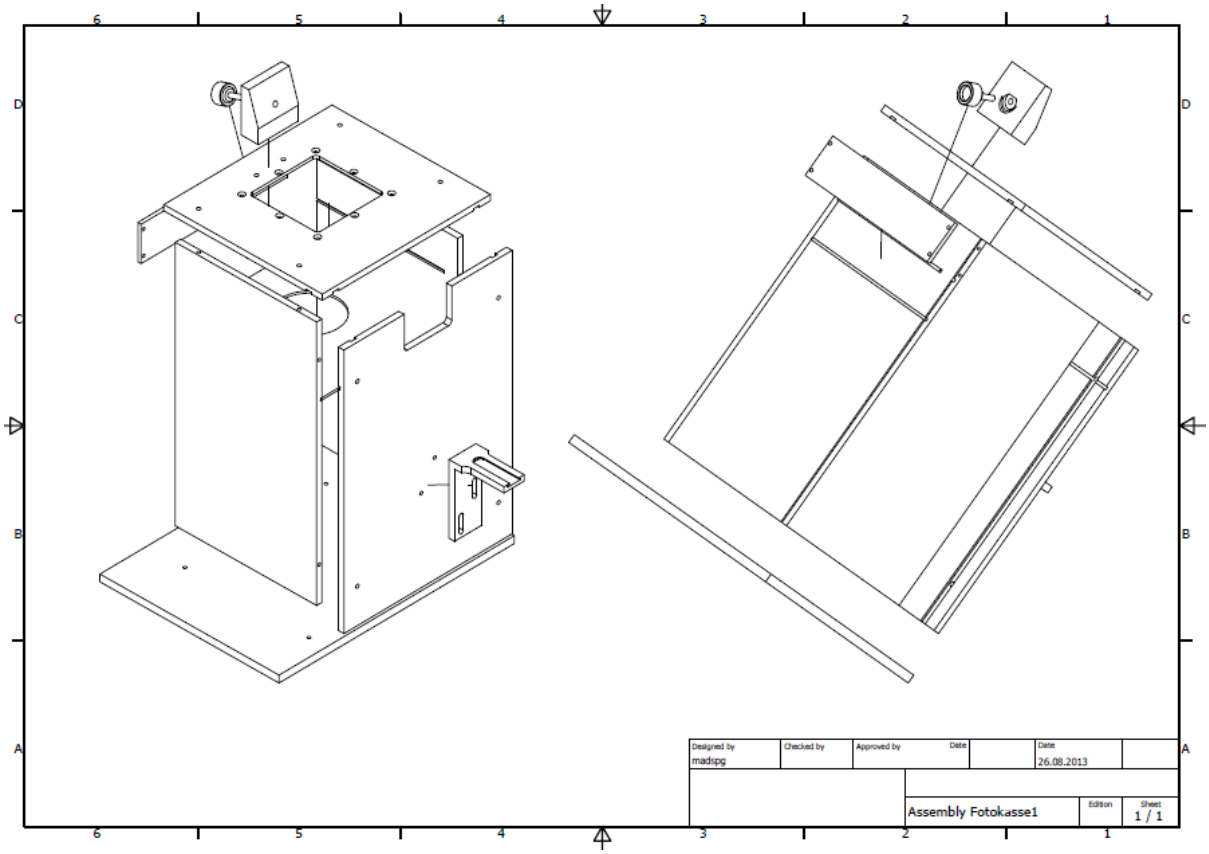
Supplementary figure 2 Reflectance spectra of crown, cheek and bib of *Passer italiae*.

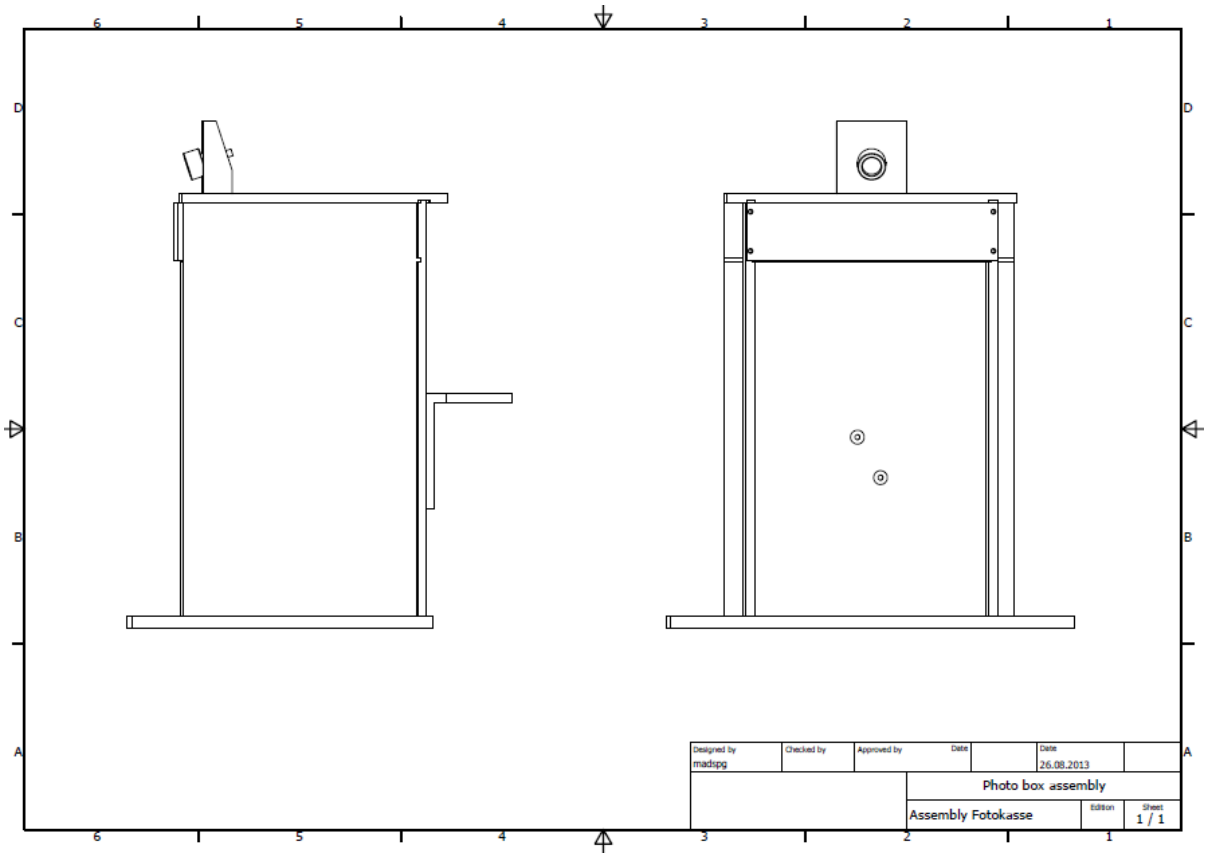


Supplementary figure 3 Reflectance spectra of crown, cheek and bib of *Passer hispaniolensis*.

Appendix 4

Technical drawings of the photo box which is included in the new method. They are shown in the following order: complete box (two views), camera mount, screw for camera mount, diffuser parts: plexiglass plate and frontal plate, right side wall, left side wall, back wall, aluminium flash mount and base plate.

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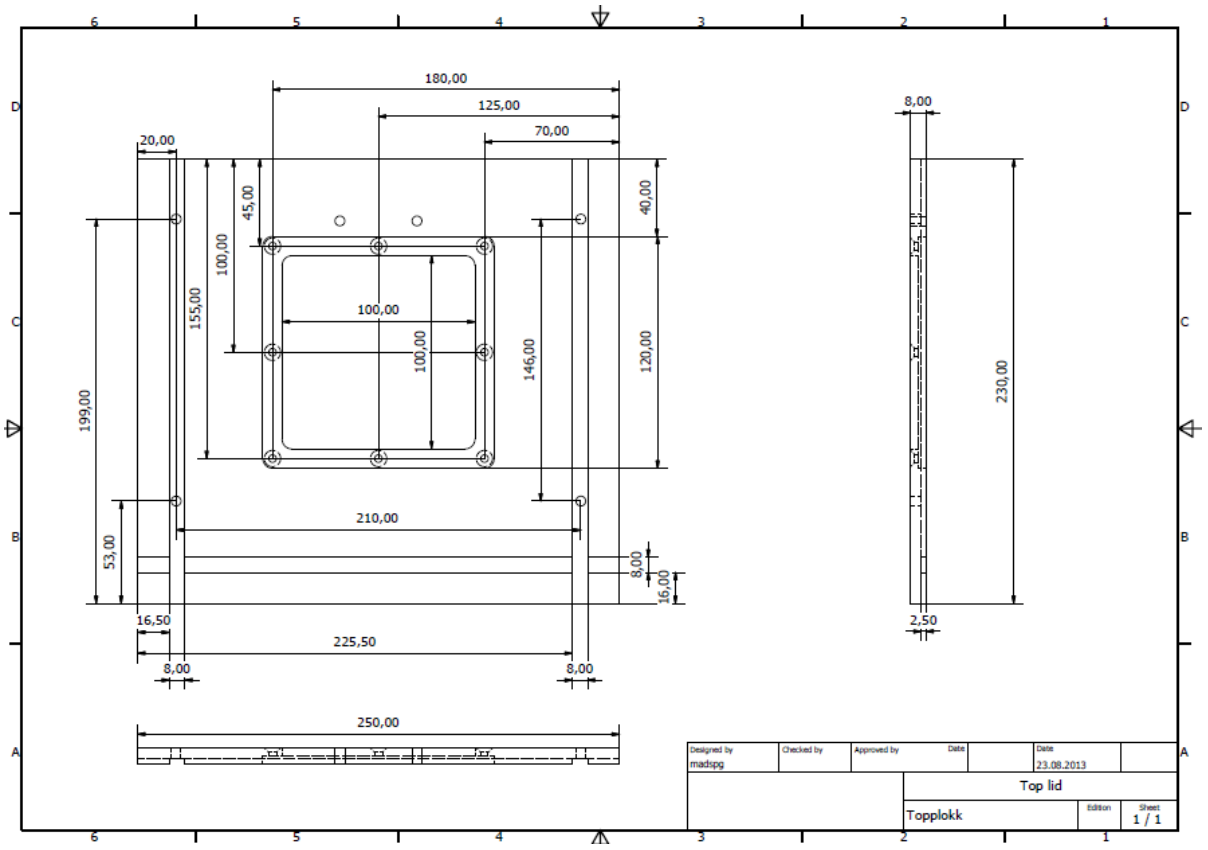
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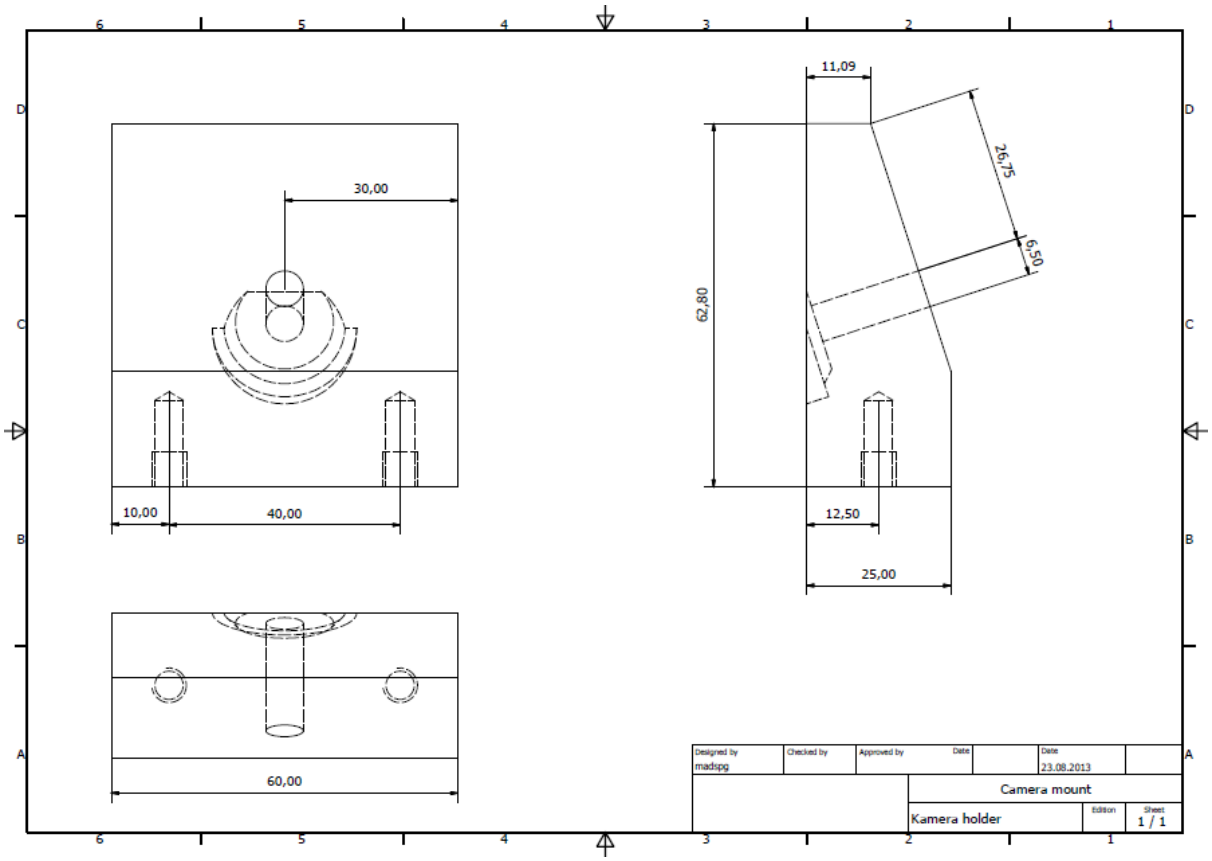
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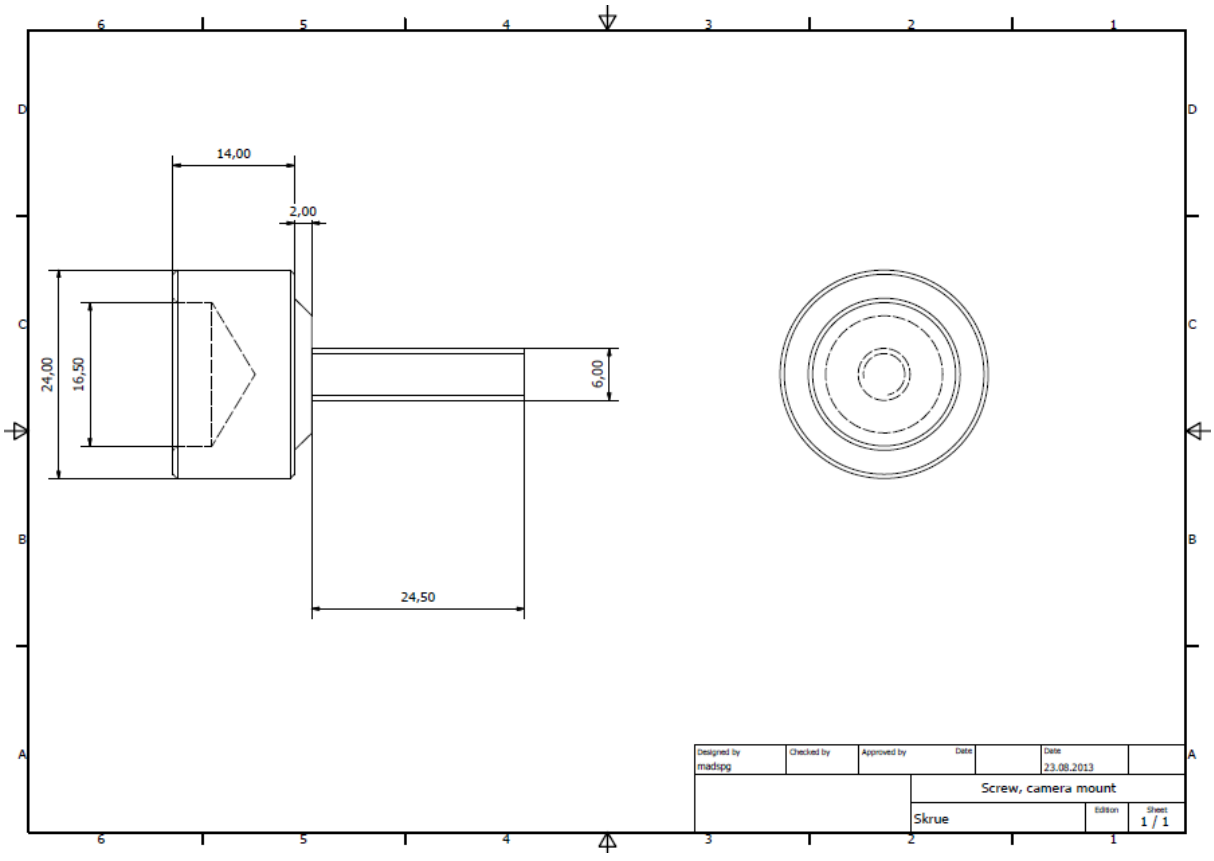
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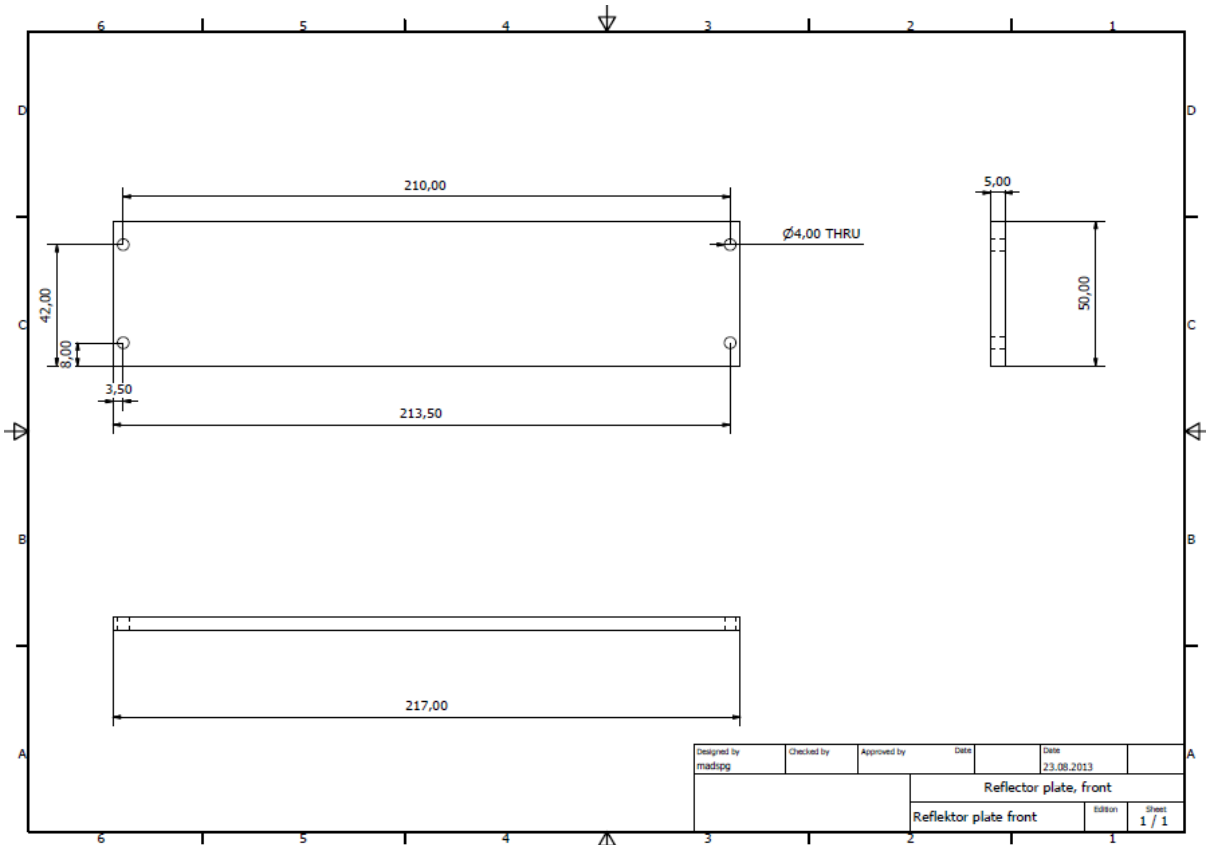
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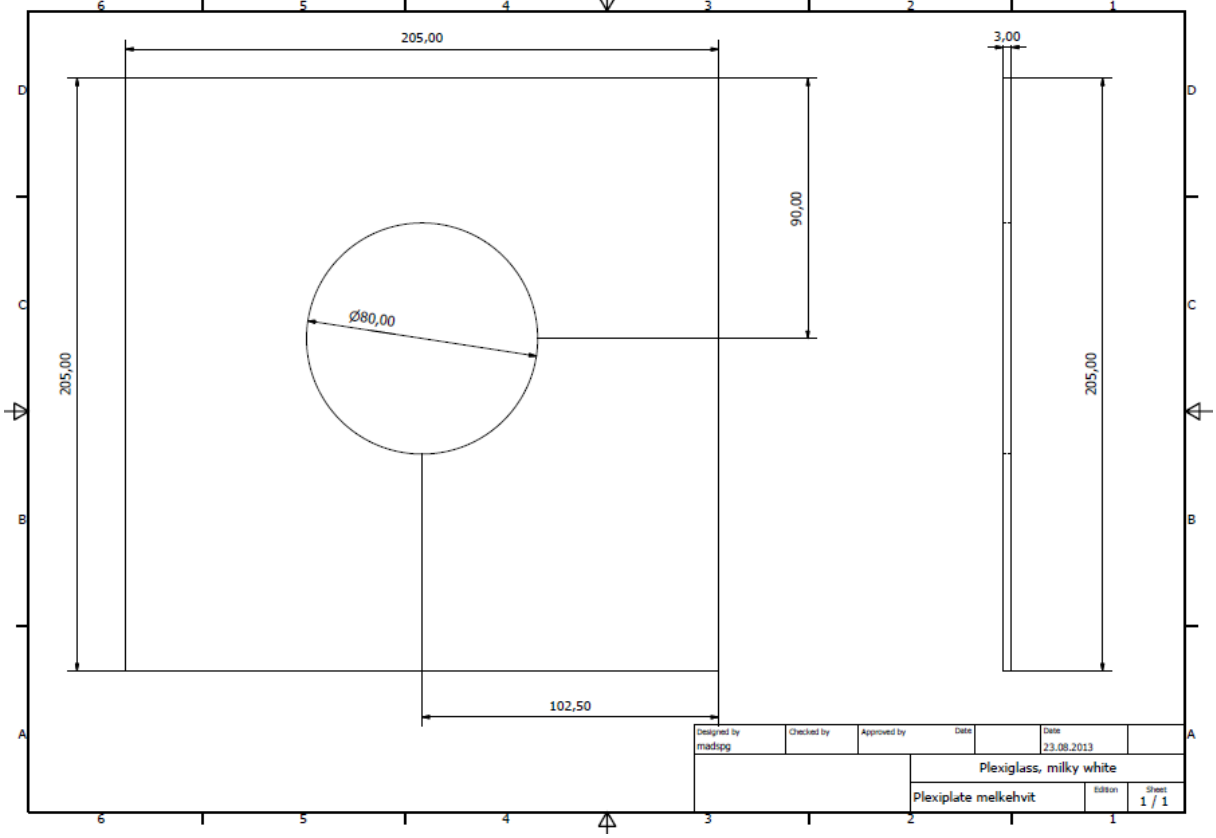
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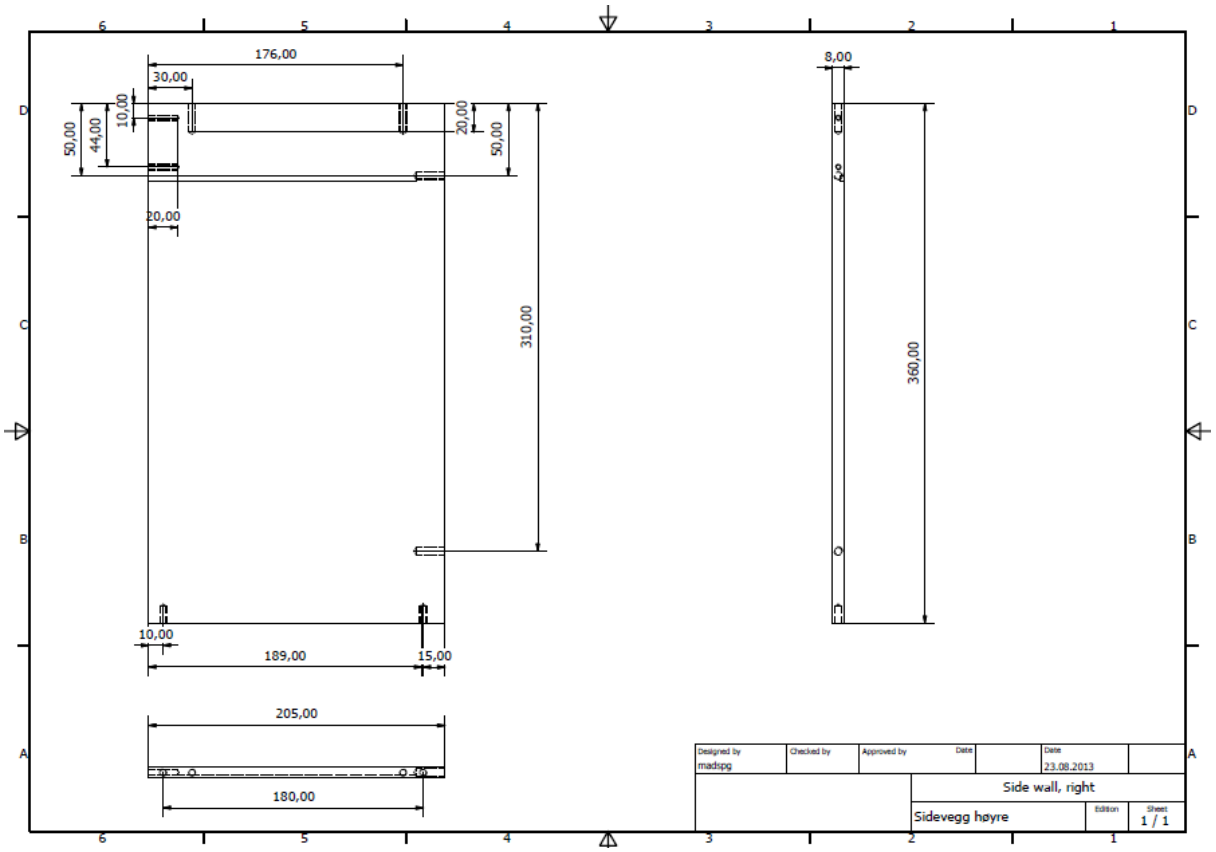
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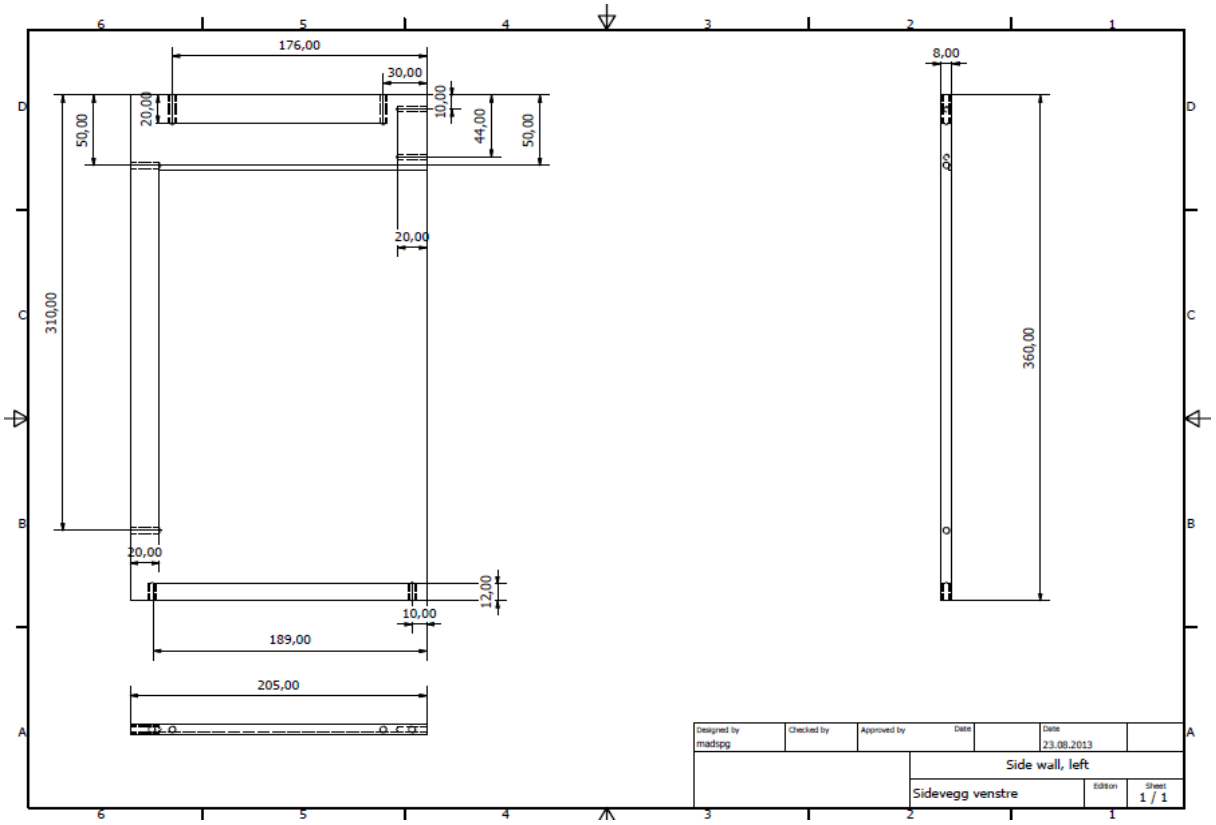
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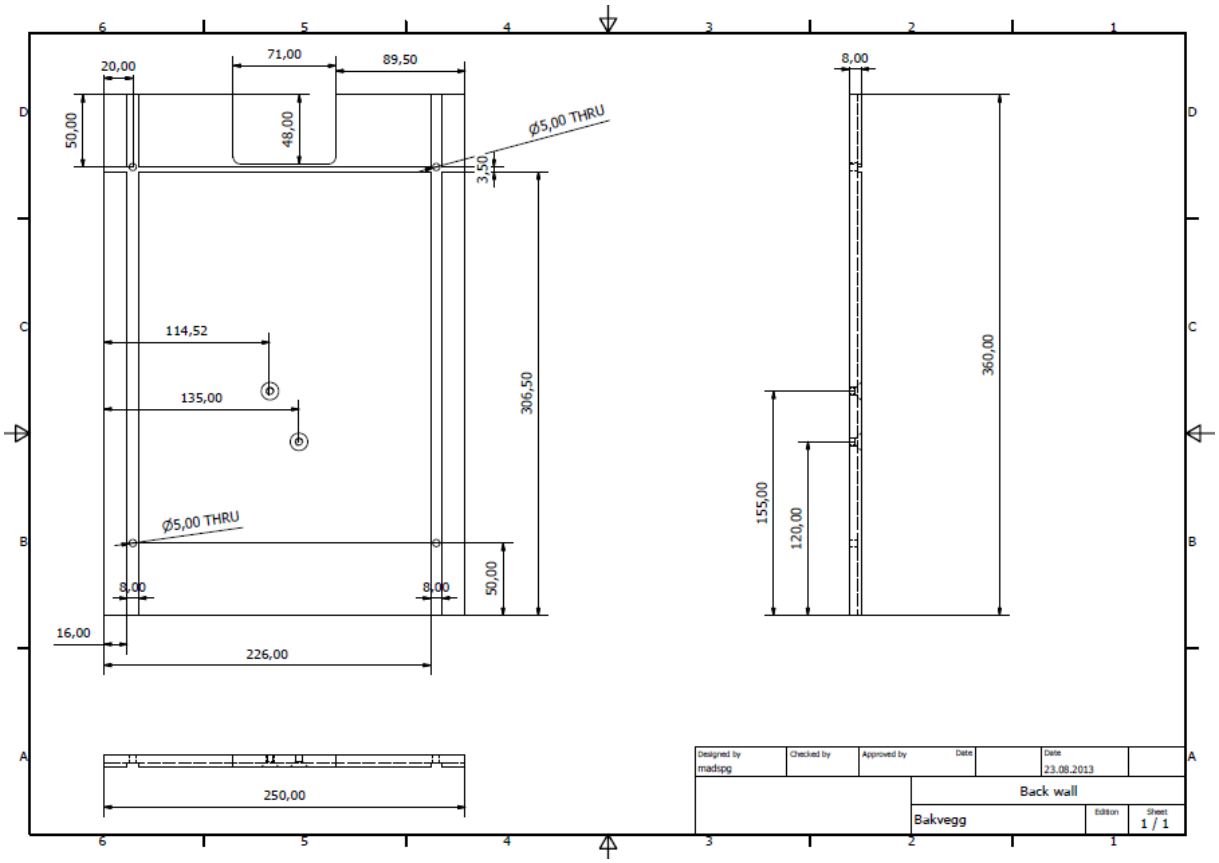
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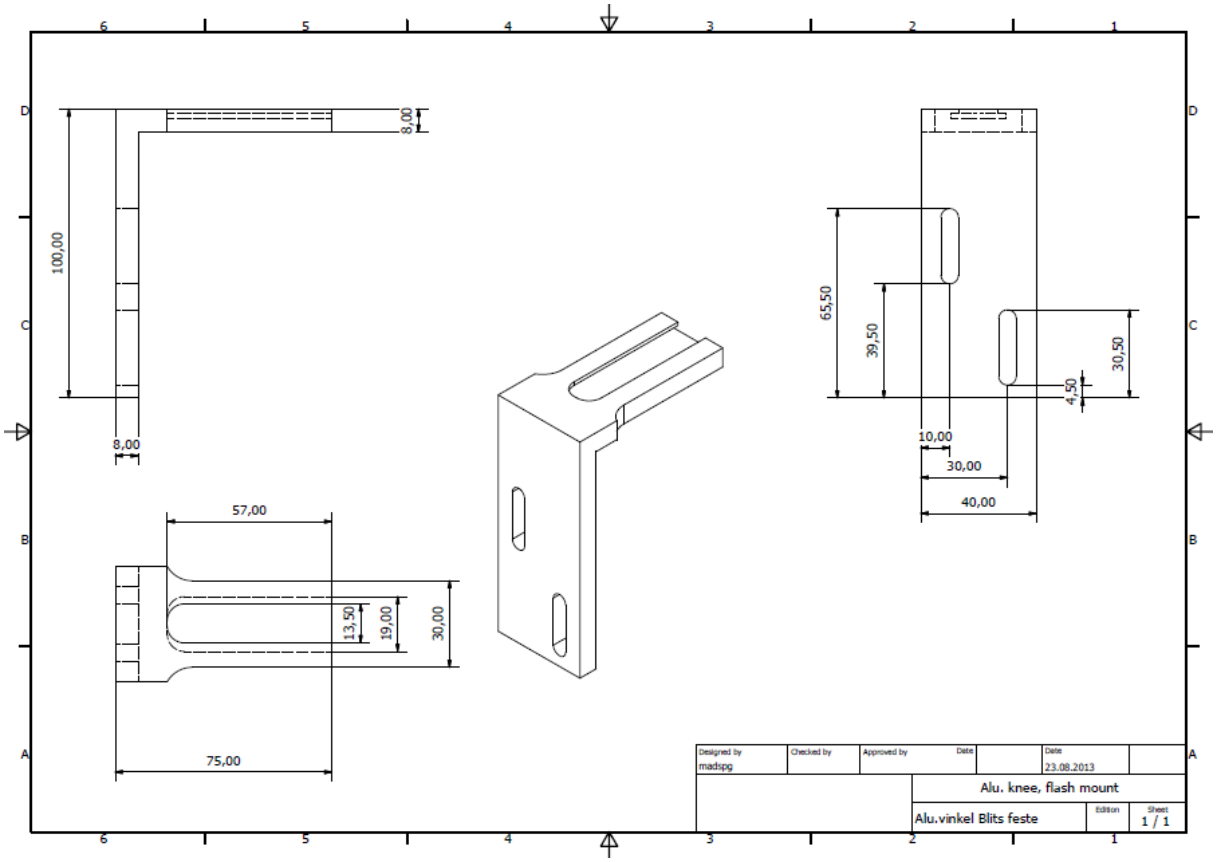
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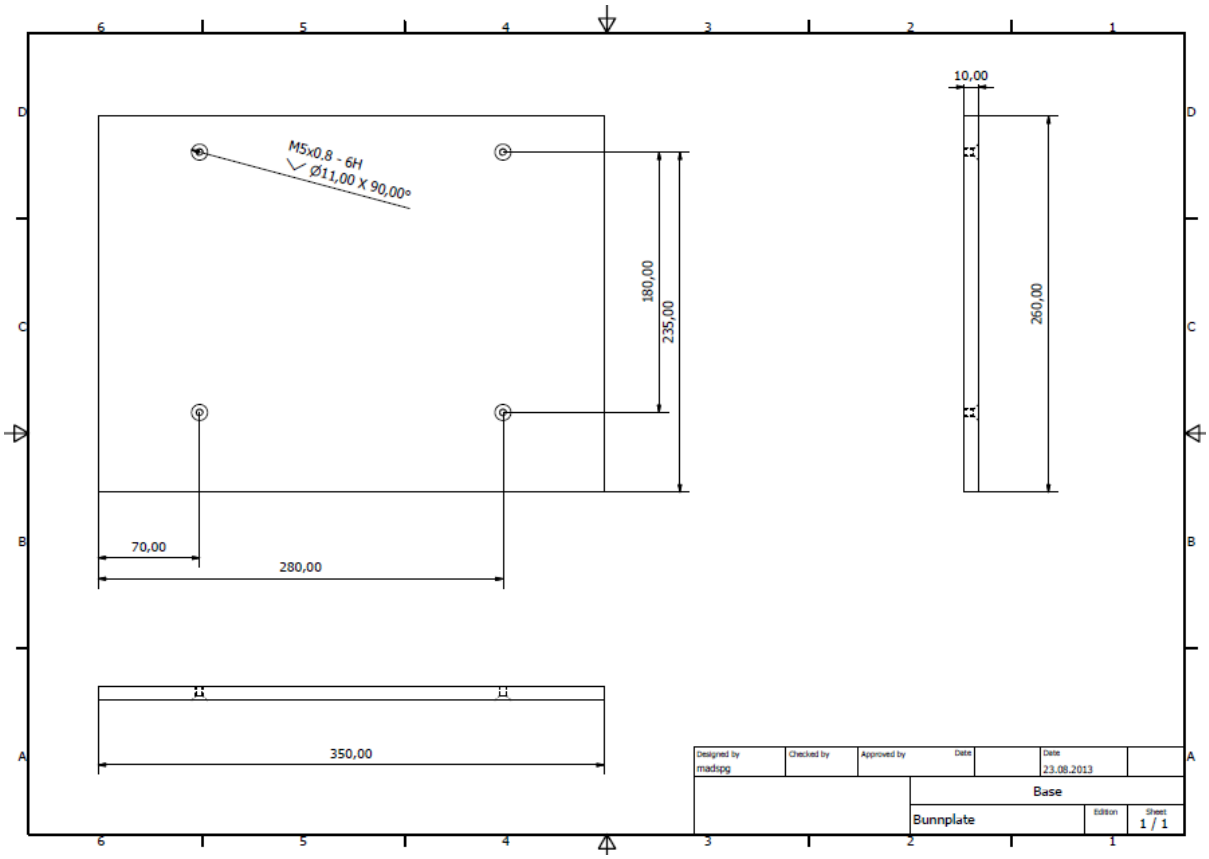
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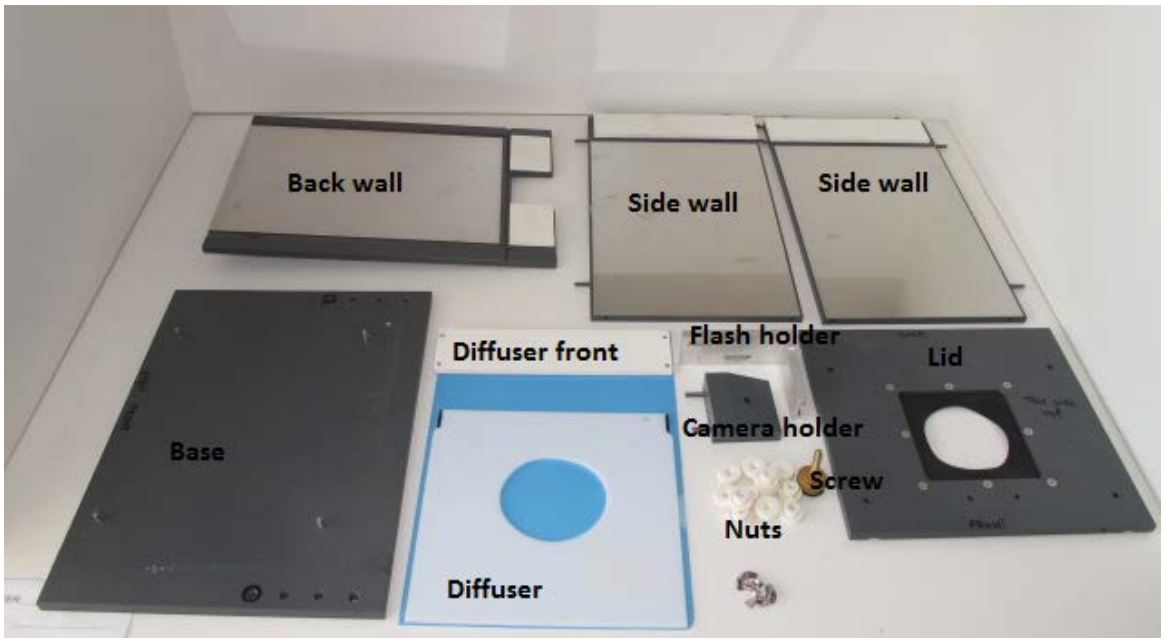
Appendix 5

PHOTO-BOX MANUAL

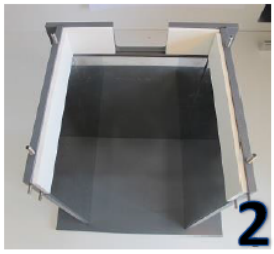
Parts

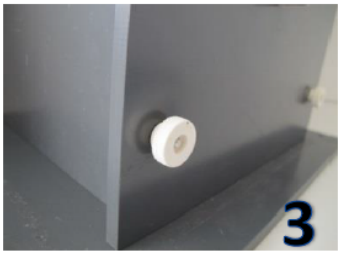
1x base
1x back wall
2x side walls
1x diffuser
1x lid
1x diffuser front
4x M4 nuts (marked red)
10x white plastic nuts
1x flash holder
2x white larger plastic nuts (marked black)
1x camera holder
1x metal screw



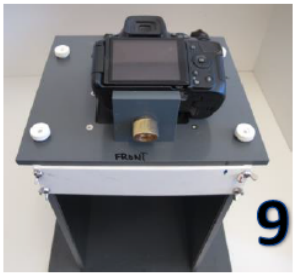
Assembly

1. Start with mounting the side walls to the base by placing the walls onto the protruding pegs.
2. Then attach the back wall.
3. Fasten with white plastic nuts in the back wall.
4. Clean the windows with spirits using cotton (paper makes scratches).





5. Attach the lid using white plastic nuts.
6. Attach the camera mount on top of the lid using **larger** (marked with black) white plastic nuts.
7. Slide the diffuser into its tracks.
8. Attach the diffuser front using **M4** nuts.
9. Attach the camera to the camera mount using the metal screw.
10. Attach the flash to the flash mount.
11. Stick the flash (with wireless receiver attached) trough the flash whole in the back wall. Attach the flash mount with the white plastic nuts in the appropriate flash-position, which is when the flash protrudes through the back wall and into the light compartment.



The box is now ready to use.

Appendix 6

FIELD PROTOCOL FOR PHOTOGRAPHY

SETTINGS

Camera and flash are set to the right settings, but it is smart to check the settings now and then.

Camera

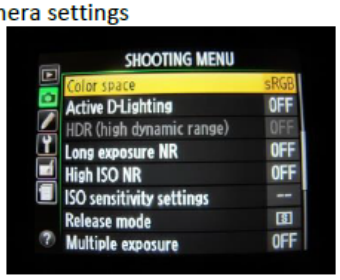
Camera screen settings

M – Manual

Adjusting exposure time	Adjusting aperture
	
Scroll wheel (1/125)	Press +/- button, then scroll wheel (F8)

Menu settings

 - Everything regarding camera settings



The lens is to be adjusted to no zoom (white mark at 10mm).
It is taped in the right position.

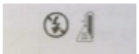
Flash

The flash uses the standard menu settings.
Screen settings

Wireless flash

Transmitter → on camera (where the flash usually is attached)

Receiver → on flash (at the bottom of the flash, the receiver is attached to the flash mount)

If the flash does not work it has gone into **standby mode**. To wake it up again press the FLASH button halfway down and it will work again. (Or you might need to change the battery.)
According to its manual, the flash needs to **cool down** for 10 minutes **after 15 times continuous** shootings, and also if this sign is showing: 

PROCEDURE

Equipment

Flash and camera: on
Transmitter (on the camera): On, press button A, set to CH1 (Channel 1)
Receiver (on the flash): On, press button A, set to CH1 (Channel 1)

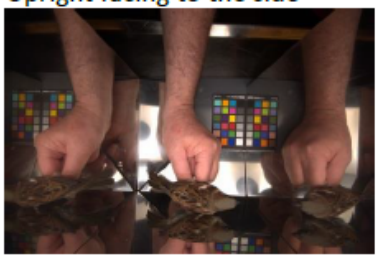
Colour checker

The colour checker should be in every photo. **We only have one colour checker**.
Be careful to avoid bird faeces and dirt.

Positions (5 different positions per bird)

The bird has to be as far into the box as possible to get the best image of all features (and less of hands) in all mirrors. Avoid hands or fingers over the colour checker, especially the grey scale.

1. Upright facing to the side



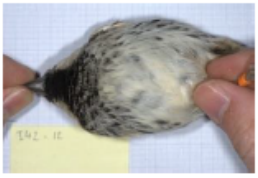
2. Upright facing into the box

3. Portrait

4. Dorsal

Back colour important

5. Ventral



Things to remember:

Minimize hands in picture as much as possible

As much features of bird in all mirrors as possible

Be careful with the colour checker

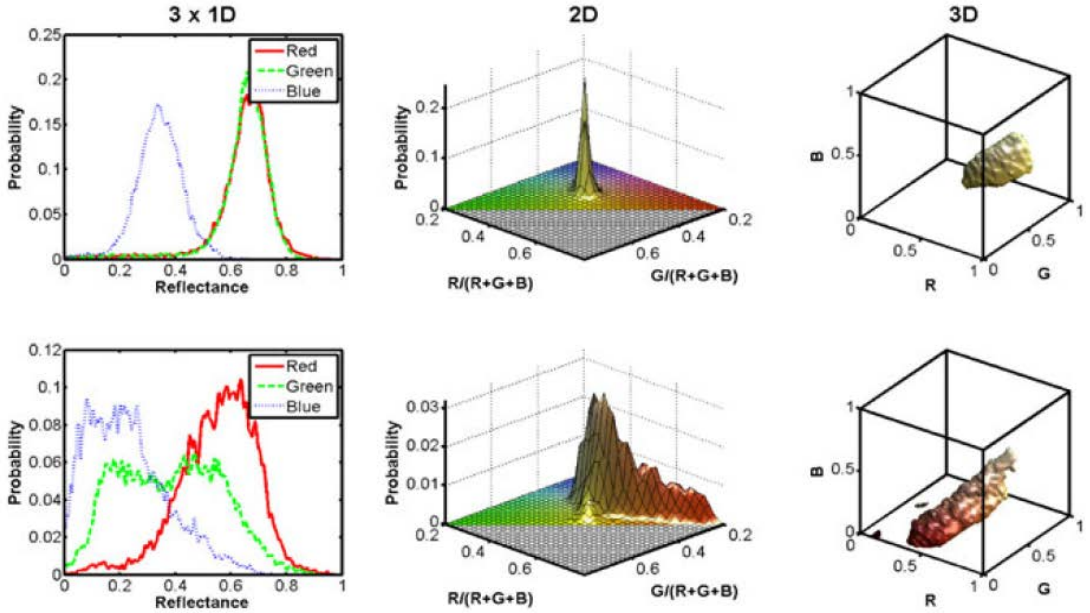
Check if the zoom is fully zoomed out

Recharge camera batteries every night

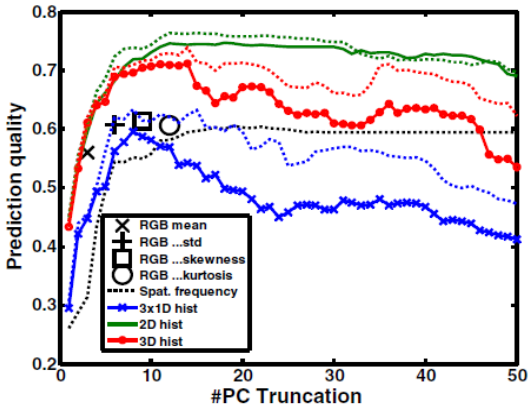
Appendix 7

The Chromatic Spatial Variance Toolbox

The chromatic spatial toolbox (Brydegaard, 2013) provides a quantitative method for classification of animal colour patterns from RGB images. In spectroscopy, there is a well-known trade-off between spectral (the ability of a sensor to define fine wavelength intervals) and spatial resolution (e. g. amount of pixels), and it is hard to compromise (Brydegaard et al, 2012). The scripts from the toolbox allows for exploitation of data from RGB images which are high in spatial (large images), but low spectral resolution, such as the RGB images of the plumage areas of the *Passer* sparrows. The method allows for utilization of the high spatial resolution in a way that uses more principal components than the number of spectral bands (three: R, G and B) and distinguish between sources using the histogram of variance rather than a single average value. The chromatic distribution of 1D, 2D and 3D histograms can vary in many ways. The data can be reduced by a Singular Value Decomposition (SVD) which is related to principal component analysis (PVA) (they both reduce a high-dimensional dataset into fewer dimension without losing important information). When running the SVD scripts, a plot of the eigenvalues are provided. The eigenvalues prior to the stagnation of decay of the eigenvalues (e. g. before the break-point)are retained for further analysis; this method is commonly referred to as Cattell's scree plot test (Cattell 1966). This approach is used to filter PCs which only contain noise. The number of PCs included in the analyses affects the performance quality (Brydegaard et al., 2012) (Supp. Figure 5), and hence selecting the correct number is important.



Supplementary figure 4 Figure 4 from Brydegaard et al. (2012) showing the one dimensional (1D), two dimensional (2D) and three dimensional (3D) probability distributions, fields and planes, respectively. The upper row shows a homogeneously coloured specimen and the lower row shows a patchy coloured specimen. The different dimensionalities allows for better exploitation of the colour information in RGB images. The figure is published with the authors' approval.



Supplementary figure 5 Figure 10 from Brydegaard et al. (2012). The figure shows the prediction quality of different models as a function of truncation (degrees of freedom minus one). This figure is included to show that various models give varying quality results and that the quality depends on the number of PC axis included (there are quality peaks). The figure is published with the authors' approval.

Additional References in the Appendices

Cattell, R. B. (1966). "The Scree Test for the Number of Factors." *Multivariate Behavioral Research*, *1*, 245-276.

Brydegaard, M., Runemark, A. & Bro, R. (2012). Chemometric approach to chromatic spatial variance. Case study: patchiness of the Skyros wall lizard. *Journal of Chemometrics*, *26*, 246-255.

Brydegaard, M. (2013). *Chromatic Spatial Variance Toolbox*. Retrieved from http://www.models.life.ku.dk/ChromatricSpatialVarianceToolbox